THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

VOLUME 180
BALTIMORE
1949

CONTENTS OF VOLUME 180

No. 1, August, 1949

No. 2, SEPTEMBER, 1949

No. 3, OCTOBER, 1949

CORRECTION

On page 171, line 22, Vol 180, No. 1, August, 1949, read *15.9* for *14.9*.

STUDIES ON THE MECHANISM OF AEROBIC PHOSPHORYLATION

By J. P. HUMMEL* AND OLOV LINDBERG

(From the Wenner-Gren Institute, University of Stockholm, Sweden)

The esterification of orthophosphate has been convincingly demonstrated to be coupled with the passage of electrons through the hydrogen transport system (1). The nicotinamide and flavin nucleotides are closely related structurally to the adenosine polyphosphates; they might well participate directly in aerobic phosphorylation. The following investigation was undertaken to study the rate of phosphate turnover in these nucleotides after incubation of a respiring liver homogenate with radioactive orthophosphate. The separation of adenylic acid (AA), adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), flavin mononucleotide (FMN), and flavin-adenine dinucleotide (FAD) from each other by ordinary chemical means is extremely difficult, especially on a small scale. We therefore took recourse to the more specific separations obtained by paper chromatography. The separations so obtained were adequate to allow rough estimations of relative specific activities of most of these components. The data presented provide strong evidence for the view that the flavin nucleotides participate in the phosphorylation which is coupled with the hydrogen transport system.

Methods and Materials

Healthy young rabbits, free of coccidiosis, were killed by decapitation. After careful removal of connective tissue, the livers were homogenized in the cold in a steel homogenizer with 2 volumes of 1 per cent potassium chloride, according to the procedure of Potter and Elvehjem (2), and with enough dilute sodium bicarbonate to maintain the final pH near 8. To 100 ml. of the cold homogenate in a 500 ml. Erlenmeyer flask were added 2 ml. of 0.1 M $MgCl_2$, 40 ml. of 0.05 M bicarbonate buffer of pH 7.8, 4 ml. of 0.25 M sodium L-glutamate at pH 7.8, and 6 ml. of 1 M NaF. This mixture was incubated for 2 minutes at 30° under vigorous aeration with 95 per cent O_2-5 per cent CO_2 mixture in order to use up any orthophosphate present; then 10 μM of phosphate buffer of pH 7.8 containing 0.4 mc. of radioactive Na_2HPO_4 (equivalent to 1.2 × 10^8 impulses per minute in the counter used) were added. The incubation and aeration were con-

1

tinued 5 minutes more, the flask contents were then chilled, and the reaction was stopped by the addition of 30 ml. of ice-cold 50 per cent trichloroacetic acid. The precipitate was filtered off at 0°, and 10 ml. of the filtrate were removed for the subsequent determination of specific activity of ATP. The remainder of the filtrate was extracted three times with 100 ml. of ether to remove the trichloroacetic acid, and the pH was cautiously adjusted to about 4.5 with 2 N NaOH. The opalescent yellow solution was saturated with solid ammonium sulfate, refiltered, and extracted with 5 ml. of water-saturated phenol according to the procedure of Crammer (3). The intensely yellow-colored phenol layer, containing the flavin and pyridine nucleotides and small amounts of ATP, was drawn off, and the solution was extracted twice with 5 ml. portions of phenol. The pooled phenol extracts were centrifuged at high speed for 10 minutes. The clear phenol solution, which could be conveniently drawn off by means of a large syringe, was washed with a mixture of 4 parts of saturated ammonium sulfate solution and 1 part of saturated potassium acid phosphate solution in order to dilute any remaining traces of radioactive orthophosphate. After separation by centrifugation, the phenol was again washed with saturated ammonium sulfate, and after high speed centrifugation, was carefully drawn off and transferred to a 200 ml. separatory funnel. The phenol-extracted nucleotides were then transferred to water by adding 10 volumes of peroxide-free ether and shaking with very small portions of water until the water extracts were colorless. The combined water extracts were dried on a watch-glass *in vacuo* over sulfuric acid. Just before analysis, they were redissolved in 0.2 ml. of water.

The procedure used for partition chromatography closely followed that originally employed by Consden, Gordon, and Martin (4) for the analysis of amino acid mixtures. Strips of Munktell's No. 8 filter paper 1.5 cm. wide and 50 cm. long were treated with 6.6 μl. of the test solution accurately measured with a calibrated micro pipette (5). The strips were hung in chromatograph chambers which were kept in the dark in rooms of uniform temperature. The following solvents were employed: (a) n-butanol-acetic acid as described by Crammer (3); (b) n-butanol-phenol (equal parts saturated with water); (c) 95 per cent pyridine; (d) phenol (water-saturated and containing 0.5 per cent 8-hydroxyquinoline); and (e) 2,4,6-collidine (water-saturated). Other solvents tested but found ineffective were dimethylaniline and furfural. When the solvent had travelled to within a few cm. of the end, the strips were removed and allowed to dry overnight in a gentle air-stream.

The location of the flavin nucleotides was observed by illuminating the strips under a "black light" mercury lamp. The areas showing a yellow-green fluorescence were outlined with pencil. Since the fluorescence of the

flavins is strongly quenched by small amounts of phenol, strips which were chromatographed with this solvent had to be washed repeatedly with xylol and finally with petroleum ether in order to develop the fluorescence.

For quantitative estimation of the flavins and their photodecomposition products on the paper, a fluorometric adaptation of the "lumiflavin" method of Warburg and Christian (6) was used. Segments of the strips 1 cm. wide were treated with 1 ml. of water and 2 ml. of 2 N NaOH in ordinary test-tubes and subjected to strong illumination by sunlight or by a photo lamp for 1 hour, with care to avoid dispersion of the cellulose in the alkaline solution by unnecessary handling. Similar segments from "empty" portions of the strip were added to the blank and to a standard containing 2 γ of riboflavin, which were treated in the same manner as the unknown samples. The samples were then made acid with 3 ml. of 2 N HCl, and the paper segments drained and discarded. The lumiflavin in the samples was extracted into 5 ml. of chloroform by vigorous shaking for 1 minute. After separation, the water layer was drawn off by suction. The blue-green fluorescence was read against the blank and the standard in a Beckman spectrophotometer with fluorometer attachment, with the blue-sensitive photocell, a 5 mm. lamp housing aperture, and with both the yellow ultraviolet opaque filter and a Corning No. 4308 filter in place.

Compounds containing adenine were located by measurement of the ultraviolet absorption of extracts of the segments at 260 mμ in the Beckman spectrophotometer. For the extraction, segments 1 cm. wide were treated with 5 ml. of 1 N HCl, and heated for 10 minutes at 100°. The absorption coefficient of adenine at 260 mμ was assumed to be 105 per mg. per ml. (7). Since phenol and pyridine seem to be firmly bound by the paper even after drying, strips treated with these solvents had to be washed in xylol repeatedly and then in petroleum ether before measuring the ultraviolet absorption. Even so, the basal absorption coefficients due to these solvents in the extracts were frequently very large. In the case of collidine chromatograms, estimation of the adenine absorption was impossible because of the very high solvent absorption.

The distribution of total phosphorus on the strips was measured by ashing segments of the paper moistened with a drop of dilute alkali in an oven at 300° for several hours. The residue was dissolved in 1 ml. of 2 N sulfuric acid and boiled 10 minutes to decompose any pyrophosphate formed. Orthophosphate was determined according to the micromethod of Berenblum and Chain (8).

The localization of DPN on the strips was accomplished in two ways. In the first, the strips were cut into 1 cm. sections; each section was clipped into pieces about 4 sq. mm., and then transferred to a Warburg flask for determination of fermentation capacity according to the method of Axelrod

and Elvehjem (9). To control the recovery from the strip, 6.6 μl. of the original test solution were added to one flask. Recoveries were never under 80 and usually about 90 per cent. The fermentation was compared with that produced by a known sample of DPN of approximately 50 per cent purity.

The second procedure was the fluorometric method of Robinson *et al.* (10), applied to 1 cm. sections of the paper strips suspended in 1 ml. of
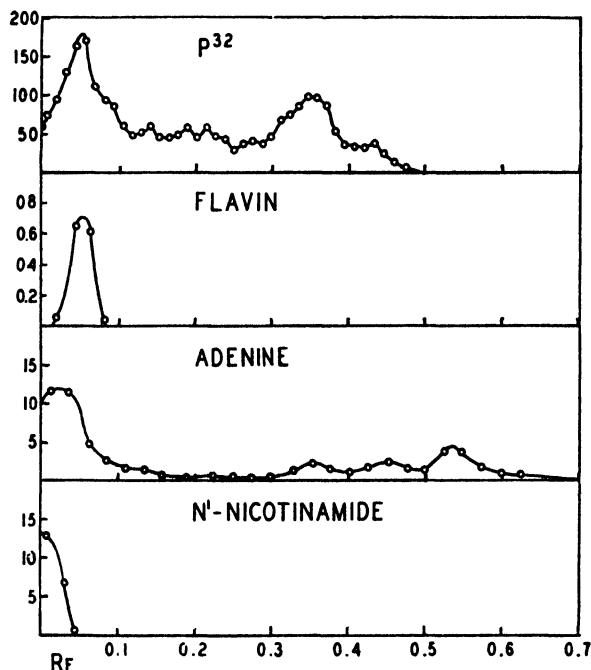
FIG. 1. The migration patterns of the phenol-soluble rabbit liver nucleotides after chromatography with the *n*-butanol-acetic acid mixture described in the text. The values for P^{32} are expressed as impulses per minute; all other data are expressed as micrograms.

water. The presence of paper containing traces of solvents did not interfere. The fluorescence of the flavins could be minimized by strong irradiation during the alkaline incubation. The fluorescence was measured against a standard quinine solution in a Beckman spectrophotometer with the fluorometric attachments previously mentioned. The method was standardized against a known solution of N^1-methylnicotinamide. The data obtained by both methods are expressed in terms of micrograms of N^1-nicotinamide.

The radioactivity on the paper was determined in the apparatus described by Lindberg and Hummel (5), having a window dimension of 5×15 mm. For determination of the specific activity of ATP, the following procedure was used. An aliquot of 10 ml. from the incubated and trichloroacetic acid-fixed filtrate was treated with 0.5 ml. of 1 M K_2HPO_4, 1 ml. of 1 M $MgCl_2$, and 1 drop of phenolphthalein. Sufficient 2.5 per cent ammonia
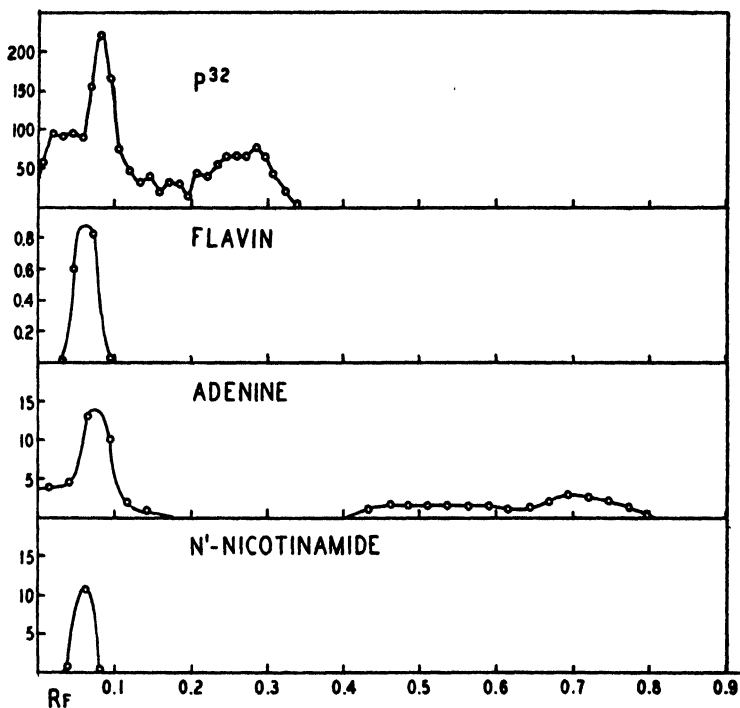
FIG. 2. The migration patterns of the phenol-soluble rabbit liver nucleotides after chromatography with the *n*-butanol-phenol mixture. The values are expressed as in Fig. 1.

solution was added to produce cloudiness. After shaking for 1 hour, the precipitate became crystalline, whereupon sufficient ammonia was added to make the solution alkaline to phenolphthalein. After filtration, the solution was again treated with 1 ml. of 1 M $MgCl_2$ and the crystallization was completed in the cold. The 7 minute-hydrolyzable phosphate and remaining orthophosphate were determined in aliquots of the refiltered solution made 1 N with hydrochloric acid.

For determination of the radioactivity in the 7 minute-hydrolyzable phosphate and in the orthophosphate, two other aliquots were taken from this

filtrate.   One was hydrolyzed for 7 minutes in 1 N HCl, then made alkaline
with ammonia, and precipitated with Na₂HPO₄ and "Fiske solution," as
described by Lindberg (11).   The other aliquot was precipitated directly
with sodium phosphate and Fiske solution.   When crystallization was
complete, the crystals were filtered off, washed with 2.5 per cent ammonia,
after which they were dissolved directly from the filter paper with 0.1 N
HCl, and diluted to a given volume.   Of this solution, aliquots were taken
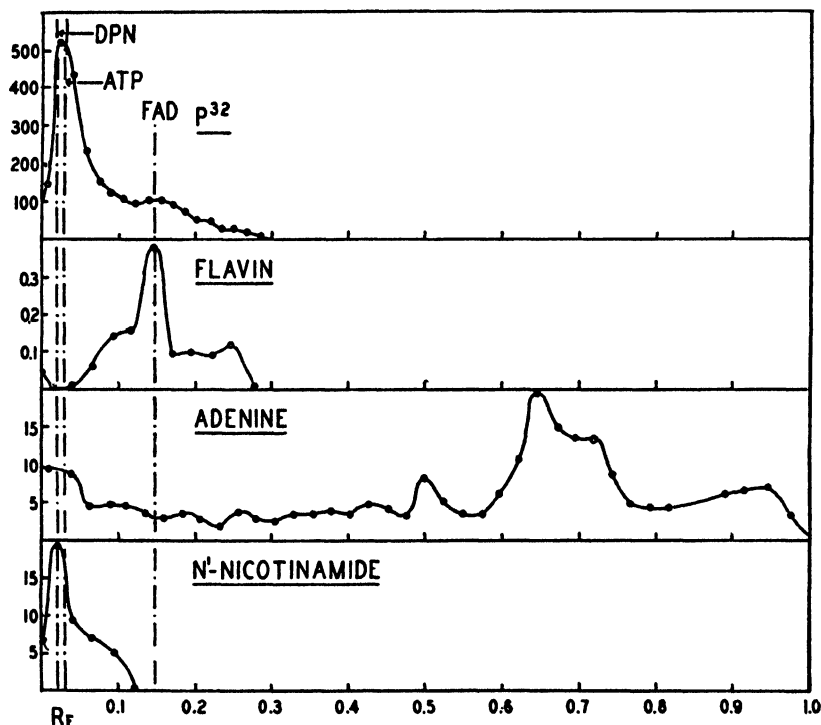
FIG. 3. The migration patterns of the phenol-soluble rabbit liver nucleotides
after chromatography with pyridine. The values are expressed as in Fig. 1.

for crystallization with Na₂HPO₄ and Fiske solution as above.   The crys-
tals thus obtained were filtered into small aluminum dishes and dried.
The purpose of these repeated crystallizations was to avoid, as much as
possible, any contamination by orthophosphate with a high specific ac-
tivity.

    Since the dimensions of the Geiger-Müller tubes used here were differ-
ent from those used with the chromatograms, suitable corrections had to
be made (5).   Under the conditions used, samples on aluminum dishes
gave values 10 per cent greater than those from the paper strips.

Pure preparations of some of the nucleotides were chromatographed with those solvents which gave more satisfactory separations in order to establish their migration constants. ATP was prepared by the method of Needham (12) as modified by DuBois *et al.* (13), DPN by the method of Sumner *et al.* (14), FAD by the method of Warburg and Christian (6, 15), and FMN by the method of Theorell (16) from old yellow enzyme as prepared by
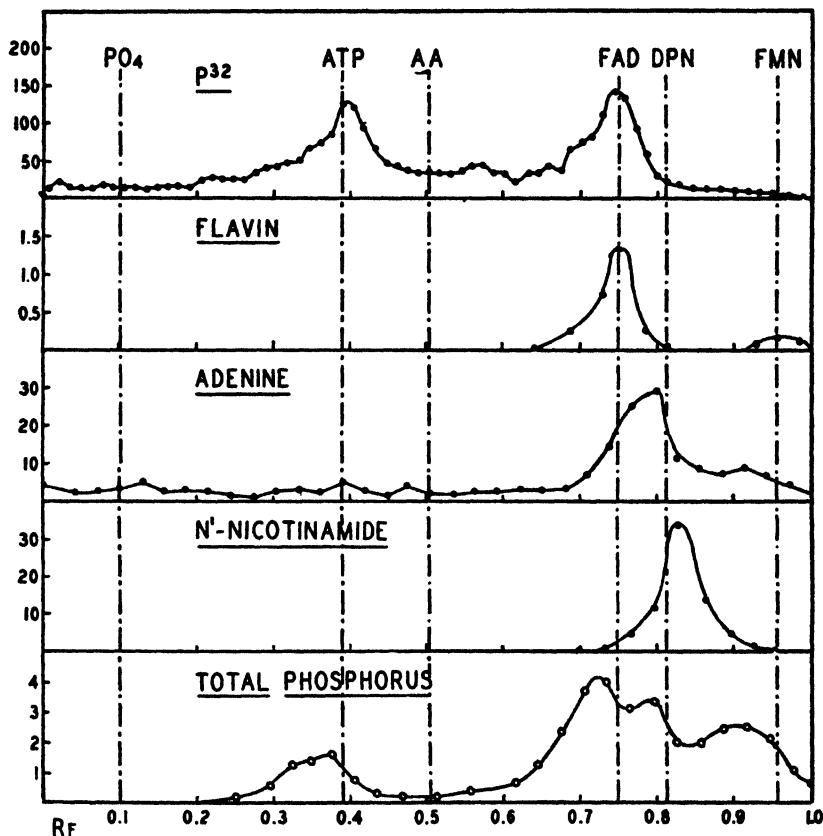
FIG. 4. The migration patterns of the phenol-soluble rabbit liver nucleotides after chromatography with phenol. The values are expressed as in Fig. 1.

Warburg and Christian (17). The AA used was prepared in Dr. J. O. Parnas' laboratory in Lwow. The radioactive orthophosphate was obtained from the United States Atomic Energy Commission.

Results

With the butanol-acetic acid mixture recommended by Crammer, the radioactivity was concentrated in two main fractions having mobilities of

0.05 and 0.35 respectively, as shown in Fig. 1. The location of the slower moving fraction coincided with the position of compounds containing flavin and adenine as determined by fluorometry and absorption spectrum respectively. The position of the N^1-nicotinamide fraction was slightly different. This solvent is therefore unsuitable for clear separations of different nucleotides. Similarly, the butanol-phenol mixture did not give sufficient migration of the nucleotides to allow any separation, as shown in Fig. 2, although the position of the radioactivity at R_r 0.08 coincided with the positions for flavin, N^1-nicotinamide, and adenine.
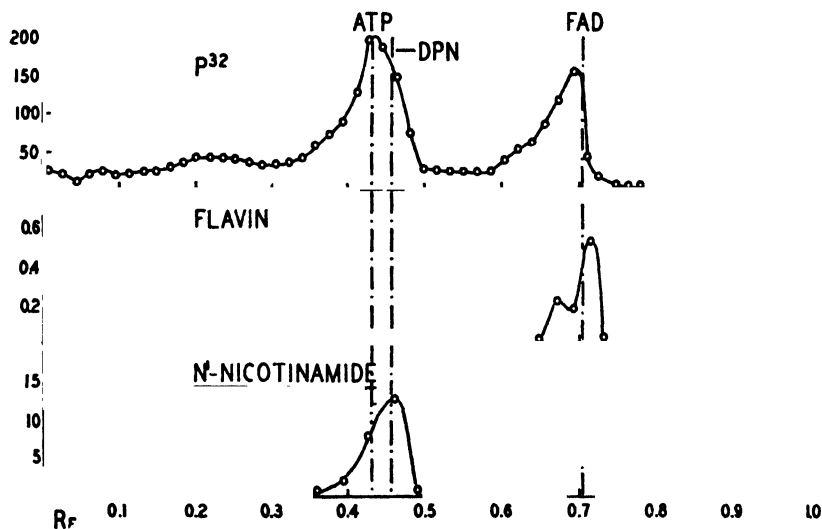
FIG. 5. The migration patterns of the phenol-soluble rabbit liver nucleotides after chromatography with 2,4,6-collidine. The values are expressed as in Fig. 1.

Pyridine, although strictly speaking not valid as a solvent for partition chromatography, since it is freely miscible with water, nevertheless gave a diffuse but significant separation of the flavin nucleotides from the adenine and nicotinamide fractions (Fig. 3). The high radioactivity in the slower band at R_r 0.03 is doubtless due to ATP in part. A small but significant peak in the radioactivity at R_r 0.15 corresponded to the position of the flavin components. A strong adenine band of high mobility was found at R_r 0.65, which had no associated radioactivity. Since pyridine has a marked alkaline reaction, possible destruction of some of the components should be kept in mind.

Phenol proved to be one of the most useful solvents for separation of the different nucleotides, despite its quenching effect on flavin fluorescence.

As can be seen in Fig. 4, two distinct peaks of radioactivity were obtained, one of which corresponds to the ATP fraction at R_F 0.39, and one to flavin-adenine dinucleotide at R_F 0.76. On the other hand, no radioactivity was found in the positions corresponding to the FMN or AA fractions. The N^1-nicotinamide band at R_F 0.83 was discrete from the flavin band, and contained no radioactivity. This was confirmed by cutting out the flavin-fluorescent area and measuring it under the counter. The adjacent region, which gave a strong DPN test by fermentation, contained little radio-activity. The adenine peak at R_F 0.80, although slightly displaced, is probably associated with the cozymase. The phosphate content in this region was high and may reflect both the DPN fraction and other phos-phorylated components of unknown nature. Certainly the minute amounts of flavin present would contribute only immeasurably to the total phosphorus.

Collidine also proved useful for the resolution of the flavin from the pyridine nucleotides. As seen on Fig. 5, the flavin fraction at R_F 0.72, which was associated with a strong radioactivity at R_F 0.70, was clearly separated from the DPN at R_F 0.46, which in this case appeared to have a migration similar to that of ATP, as shown by the high radioactivity at R_F 0.43.

Liver homogenates incubated with 95 per cent N_2-5 per cent CO_2 mixture but otherwise treated the same as those described above yielded chromatographs which contained negligible amounts of radioactivity. This shows that esterification of phosphate by glycolytic processes was not a factor in the phosphate turnover of the nucleotides under these conditions.

DISCUSSION

In all cases, radioactivity was closely identified with the ATP. In addition, radioactivity was always found in the position of the FAD fraction, with all the five solvents used. Indeed, assuming that only one of the two phosphate groups of FAD has a high turnover rate, a calculation based on the amount of flavin recovered from the phenol chromatogram gave 3500 impulses per microgram of phosphorus per minute. The specific activity of the easily hydrolyzable phosphate of ATP in the same sample was approximately 5400 impulses per microgram of phosphorus per minute. The errors of the analytical methods do not permit an exact comparison of these specific activities, but since the values are roughly of the same order of magnitude, the participation of FAD in aerobic phos-phorylation is strongly suggested, although not necessarily proved. The mechanism by which the esterification of orthophosphate takes place remains a matter of conjecture.

These data show that DPN did not take part in any phosphate exchange, since this fraction was independent of radioactivity in the phenol chromato-

grams. Moreover, the amounts of DPN present in the sample were very large compared with the amounts of FAD found, so that any uptake of P^{32} should have been easily detectable. The turnover rate of DPN *in vivo*, however, may be appreciable, as suggested by the rapid assimilation of labeled orthophosphate into DPN by respiring yeast cultures (18). It is noteworthy that only DPN was found in these samples; other nicotinamide-ribose nucleosides or nucleotides could not be detected by the chemical fluorescence method employed. It seems unlikely that these nicotinamide derivatives should have the same migration constants as DPN in five different solvents. A more plausible explanation for the absence of these simpler fragments is that the homogenate contains highly active degradative enzymes which break down the nicotinamide-ribose moiety.

The application of paper chromatography to the study of phosphate turnover may find wider application as more refined methods are discovered for the qualitative separation of nucleotides, sugar phosphates, and other phosphorylated intermediates. It affords the possibility of isolating hitherto unknown intermediates and may greatly simplify the study of functional oxidative phosphorylation.

SUMMARY

The phosphate turnover of various nucleotides in a concentrated respiring liver homogenate during incubation with radioactive orthophosphate was investigated. A method is described for the extraction of the flavin nucleotides, pyridine nucleotides, and adenylic acid from a protein-free filtrate. The nucleotides so obtained were separated by means of paper chromatography with several different solvents.

The flavin mononucleotide, pyridine nucleotides, and adenylic acid fractions contained no appreciable radioactivity. Only those fractions corresponding to adenosine triphosphate and flavin-adenine dinucleotide were uniformly radioactive. The specific activity of flavin-adenine dinucleotide was large, suggesting that the flavin enzymes are intimately associated with aerobic phosphorylation.

BIBLIOGRAPHY

1. Friedkin, M., and Lehninger, A. L., *J. Biol. Chem.*, **174**, 757 (1948).
2. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
3. Crammer, J. L., *Nature*, **161**, 349 (1948).
4. Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).
5. Lindberg, O., and Hummel, J. P., *Ark. Kemi*, **1**, No. 4 (1949).
6. Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 150 (1938).
7. Hotchkiss, R. D., *J. Biol. Chem.*, **175**, 315 (1948).
8. Berenblum, I., and Chain, E., *Biochem. J.*, **32**, 295 (1938).
9. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **131**, 77 (1939).

10. Robinson, J., Levitas, N., Rosen, F., and Perlzweig, W. A., *J. Biol. Chem.*, **170,** 653 (1947).
11. Lindberg, O., *Ark. Kemi, Mineral. o. Geol.*, **23** A, No. 2 (1946).
12. Needham, D. M., *Biochem. J.*, **36,** 113 (1942).
13. DuBois, K. P., Albaum, H. G., and Potter, V. R., *J. Biol. Chem.*, **147,** 699 (1943).
14. Sumner, J. B., Krishnan, P. S., and Sisler, E. B., *Arch. Biochem.*, **12,** 19 (1947).
15. Warburg, O., and Christian, W., *Biochem. Z.*, **298,** 368 (1938).
16. Theorell, H., *Biochem. Z.*, **275,** 344 (1935).
17. Warburg, O., and Christian, W., *Biochem. Z.*, **266,** 377 (1933).
18. Lindahl, P. E., Malm, M., Strindberg, B., and Lagergren, B. M., *Nature*, **158,** 746 (1946).

THE OXALACETATE DECARBOXYLASE OF AZOTOBACTER VINELANDII*

BY G. W. E. PLAUT AND HENRY A. LARDY

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

In 1941 Krampitz and Werkman (1) discovered an enzyme in *Micrococcus lysodeikticus* which catalyzed the decarboxylation of oxalacetate. By means of an exchange reaction they were able to demonstrate the incorporation of isotopic carbon dioxide into the β-carboxyl group of oxalacetic acid in the presence of this enzyme (2). Similar results were obtained by Evans, Vennesland, and Slotin (3) with an enzyme system from pigeon liver. Further studies on the C_3—C_1 condensation have been reviewed by Wood (4) and will, therefore, not be discussed here.

The apparent importance of oxalacetate decarboxylase as one of the gateway enzymes for the fixation of carbon dioxide into compounds of the Krebs isocitric acid cycle made it of interest to attempt to purify this enzyme and to study some of its properties. The biotin content of the enzyme fractions at various stages of purity was determined in view of the recent implication of biotin in the metabolism of carbon dioxide (5–11).

Lee, Burris, and Wilson (12) demonstrated that cell-free preparations of *Azotobacter vinelandii* have oxalacetate decarboxylase activity. Since this organism is easily grown on a large scale (13), cell-free extracts of this bacterium were used as the source material for this study.

EXPERIMENTAL

Purification of Enzyme

Azotobacter vinelandii was grown on the nitrogen-free medium of Lee and Burris (13) in 250 liter quantities with vigorous agitation and aeration at 30° in 100 gallon iron tanks. After 24 hours the cells were harvested with a Sharples centrifuge. Usually 400 to 600 gm. of cell paste were obtained. The bacterial centrifugate was suspended with vigorous agitation in 6 liters of acetone at 5°. The suspension was filtered with suction. The filter cake, still wet with acetone, was resuspended in 6 liters of cold acetone with vigorous agitation. After filtration with suction the residue was placed in a vacuum desiccator containing calcium chloride and shaved

paraffin and was dried at 10° *in vacuo* for 10 to 15 hours. In various runs 100 to 150 gm. of dried cell powder were thus obtained.

The progress of the purification was followed with the method subsequently described for measuring enzymatic activity. Nitrogen was determined by a micro modification of the direct nesslerization procedure of Koch and McMeekin. All specific enzyme activities were expressed as microliters of CO_2 per 15 minutes per microgram of N at 30°.

In a typical experiment the following procedure was used. 20 gm. of acetone-dried cells were ground to a fine powder and then stirred vigorously for 2 hours in 400 ml. of distilled water at 5°. The suspension was centrifuged in an angle head centrifuge at high speed at 5° until the supernatant was only faintly turbid. The supernatant enzyme solution had a specific activity of $1 \mu l$. of CO_2 per microgram of N. The residue was discarded and the solution was chilled in an ice bath and acidified with 1 N hydrochloric acid to pH 4.8. The heavy precipitate thus formed was centrifuged off at 5°. 300 ml. of a clear orange solution with a specific activity of 7 μl. of CO_2 per microgram of N was obtained with a yield of 80 per cent of the enzyme activity.

The acid solution was next treated at 5° with 240 ml. of approximately 1 M copper hydroxide gel. After very gentle agitation for 5 minutes the suspension was centrifuged in the cold for 5 minutes. The supernatant, pH 5.8, contained practically no activity and was discarded. The copper precipitate was *immediately* suspended in 240 ml. of cold 0.1 M phosphate buffer of pH 6.8 and stirred for 10 minutes. The suspension was *immediately* centrifuged in the cold. The light orange supernatant solution had a specific enzyme activity of 14 μl. of CO_2 per microgram of N. The recovery for the copper step was 50 per cent. However, the contact time with copper hydroxide during adsorption and elution had a profound effect on enzyme recovery and the purity of the final product. Longer adsorption times led to loss of activity, while shorter contact with the copper gel was less effective in removing impurities.

The eluate was dialyzed with rapid agitation against three changes of distilled water at 10° for 150 minutes and was subsequently lyophilized.

The tan-colored powder was dissolved in 30 ml. of cold 0.01 M ammonium hydroxide. The turbidity which consisted mainly of residual copper hydroxide was centrifuged off and discarded.

27 ml. of saturated ammonium sulfate[1] were added to 30 ml. of solution. Upon centrifugation in the cold the residue was discarded, since it had a specific activity of only 3 to 4 μl. of CO_2 per microgram of N and constituted only 10 per cent of the eluate activity. The supernatant solution

---

[1] Saturated at room temperature (*ca.* 23°) and containing 0.02 M ammonium hydroxide.

was more completely saturated by the dropwise addition, with stirring, of 43 ml. of saturated ammonium sulfate solution. After centrifugation the supernatant solution was discarded, since it contained practically no activity. The residue was dissolved in 20 ml. of cold succinate-borate buffer, pH 6.8,[2] and dialyzed for 150 minutes with strong agitation against two changes of 8 liters of distilled water at 10°. The enzyme solution was lyophilized. The pink powder usually had a specific activity of 30 to 35 μl. of CO_2 per microgram of N and contained 50 to 60 per cent of the activity present in the phosphate eluate of the copper hydroxide gel.

The powder was dissolved in 20 ml. of cold 0.01 M ammonium hydroxide and 17 ml. of saturated ammonium sulfate were added. The low potency precipitate was discarded and the supernatant solution was more completely saturated with 30 ml. of ammonium sulfate solution.[1] The residue obtained upon centrifugation dissolved completely in 20 ml. of succinate borate buffer, pH 6.8. After dialyzing for 150 minutes against two changes of 8 liters of distilled water at 10°, the solution was lyophilized. A pink powder with a specific activity of 40 to 80 μl. of CO_2 per microgram of N was obtained.[3] The yield in this step was 60 to 70 per cent.

Method of Determination

The procedure employed to test for oxalacetate decarboxylase activity was essentially a modification of that used by Straub (14) and Krampitz and Werkman (1).

The reaction was carried out in Warburg vessels with two side arms. The enzyme solution, 0.01 ml. of 0.2 M $MnSO_4$, and sufficient 0.1 M buffer[2] at pH 6.8 to bring the final fluid volume to 3.0 ml. were placed in the main compartment of the vessels. The substrate (0.2 ml. of 0.1 M oxalacetic acid) was placed in one side arm and 0.2 ml. of 10 N H_2SO_4 in the other. Deviations from this mixture will be so indicated in the text. The oxalacetic acid was always dissolved in sufficient 0.134 N NaOH to give a final concentration of 0.1 M oxalacetate. These solutions were prepared immediately before use and added to the flasks just before attaching them to the manometers. The flasks were incubated for 5 minutes at 30° with the stop-cocks open, the stop-cocks were closed, and the equilibration was continued for 10 minutes. At the end of this time the initial reading was taken. The substrate was then dumped into the main compartment. The sulfuric acid was dumped from the other side arm 15

[2] Succinate-borate buffer was prepared by mixing 500 ml. of 0.1 M sodium succinate and 500 ml. of 0.1 M boric acid; the solution was adjusted to pH 6.8 by the addition of 0.1 N HCl.

[3] All enzyme preparations were analyzed for free ammonia nitrogen and, when present, this was subtracted from the total nitrogen of the preparation.

minutes later to stop the enzymatic reaction and to liberate the carbon dioxide retained as bicarbonate by the buffer. The final readings were then taken. A boiled enzyme blank was run with all determinations to account for the chemical decomposition of the substrate. This blank value (identical with no enzyme blank) was subtracted from the total gas evolved.

Previous investigators (14–20), particularly those working with animal tissues, have reported the presence of a "heat-stable" substance which,
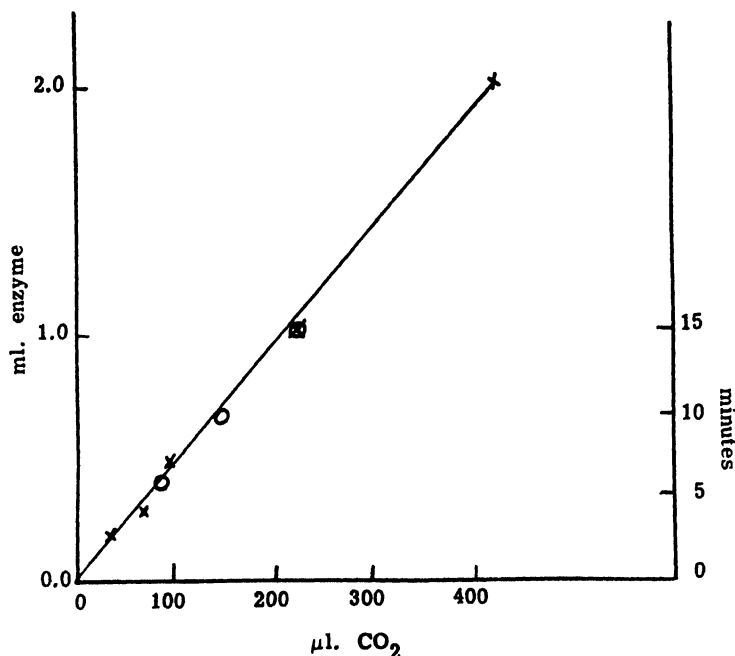
Fig. 1. Enzyme concentration-time relationship. ×, activity *versus* quantity of enzyme; time, 15 minutes; ○, activity *versus* time; 1.0 ml. of enzyme. Enzyme preparation, extract of bacterial acetone powder; 0.00067 M Mn^{++} present; temperature 30°.

even after boiling, catalyzed the decomposition of oxalacetate. Such a material was never found in *Azotobacter* preparations. Crude cell extracts contained a trace of pyruvic decarboxylase activity which disappeared after the first purification step.

The validity of the method as an assay procedure is demonstrated by the results in Fig. 1. There is a linear relationship between enzyme concentration and amount of gas evolved and likewise between time and gas evolved. Hereafter all activities quoted in this paper are expressed as microliters of CO_2 per 15 minutes at 30°.

Determination of the pH optimum revealed a wide range of maximum activity of the enzyme between pH 6.5 and pH 8. The chemical decomposition of oxalacetate increases in a linear fashion from pH 6 to pH 8.4 (Fig. 2).
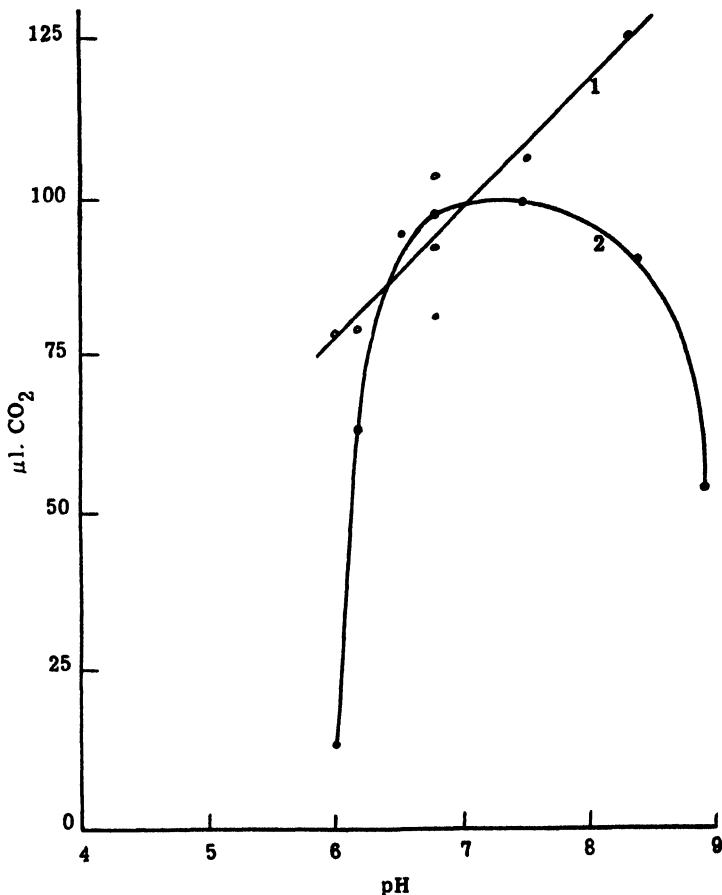
FIG. 2. Effect of pH on oxalacetate decarboxylation at 30°. Curve 1, chemical decomposition; Curve 2, net enzymatic decomposition. Specific enzyme activity, 28 μl. of CO_2 per microgram of N per 15 minutes; 0.00067 M Mn^{++} present.

Activators

Krampitz and Werkman (1) reported that magnesium and manganese were activators for the enzyme from *Micrococcus lysodeikticus*, while Evans *et al.* (3) found Mn^{++} to be the activator of choice for the pigeon liver enzyme. Vennesland and Felsher (19) obtained an increase of parsley root enzyme activity by the addition of Mn^{++} or Mg^{++}.

In much of our earlier work 0.00067 M Mn^{++} was used to activate the *Azotobacter* enzyme after it was found that this level allowed maximum enzyme activity (Table I). Several other metals were subsequently tested. Co^{++} (Tables I and II) and Zn^{++} were found to be active, Mg^{++} had slight activity, while Ni^{++} and Fe^{++} were inert (Table II). Zn^{++}, Ni^{++}, and,

TABLE I

Activation of Azotobacter Oxalacetate Decarboxylase by Co^{++} and Mn^{++}

Specific activity of enzyme, 14 μl. of CO_2 per microgram of N per 15 minutes. The data represent average values of two similar experiments.

Experiment No.	Concentration of activator		Activity
	MnSO₄	CoCl₂	
	M	*M*	μl CO_2
1	0.00067		156
	0.00201		156
	0.00335		167
2			39
		0.00017	222
		0.00034	204
		0.00067	200
	0.00067		196
	0.00067	0.00008	198
	0.00067	0.00017	212

TABLE II

Metal Activation of Oxalacetate Decarboxylase

Incubated 15 minutes at 30°. The enzyme preparation was dialyzed against distilled water. Specific enzyme activity, 2 μl. of CO_2, per microgram of N per 15 minutes.

Activator	Chemical decomposition	Net enzymatic decomposition
	$\mu l.$ CO_2	$\mu l.$ CO_2
None..........	62	21
0.00067 M Mn^{++}.	62	203
0.00017 " Co^{++}	60	225
0.00067 " Zn^{++}....	152	158
0.00067 " Mg^{++}........... .	63	56
0.00017 " Ni^{++}	101	27
0.00067 " Fe^{++}	63	22

in higher concentrations, Co^{++} increased the rate of chemical decomposition.

Inhibitors

Utter and Wood (21) demonstrated that adenosine triphosphate (ATP) increased the fixation of CO_2 into oxalacetate by pigeon liver extracts.

Vennesland *et al.* (20) purified this enzyme of pigeon liver. In agreement with Utter and Wood they obtained a stimulation of carbon dioxide fixation in the presence of ATP and also observed an inhibition by triphos-

TABLE III

Effect of Adenylic Acid upon Azotobacter Oxalacetate Decarboxylase

Specific enzyme activity, 1 μl. of CO_2 per microgram of N per 15 minutes.

Buffer system	Additions	Activity
		μl. CO_2 per ml. enzyme
0.1 M phosphate, pH 6.8..	None	108
0.1 " " " 6.8.....	0.002 M muscle adenylic acid	110
0.1 " succinate-borate, pH 6.8......	None	1650
0.1 " " " 6.8......	0.002 M muscle adenylic acid	1620
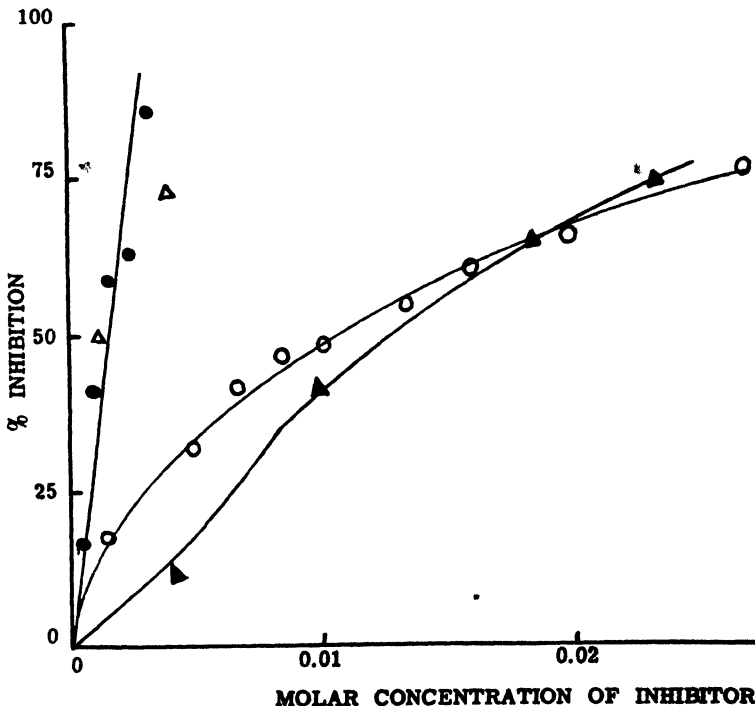
FIG. 3. Inhibition of oxalacetate decarboxylase. 0.1 M succinate-borate buffer pH 6.8, and 0.00067 M manganous sulfate. ●, pyrophosphate; △ adenosine triphosphate; ○, orthophosphate; ▲, L-malate.

phopyridine nucleotide. The decarboxylating activity of pigeon liver was found to be inhibited by ATP and stimulated by triphosphopyridine nucleotide.

It seemed possible from the foregoing that adenylic acid, acting as phosphate acceptor, might stimulate the rate of decarboxylation of the *Azotobacter* enzyme. This was found not to be the case. The possibility was next considered that the stimulating activity of adenylic acid could be demonstrated in the presence of low phosphate concentrations. The 0.1 M phosphate buffer of pH 6.8 which had been used in the studies up to that time was, therefore, replaced by a 0.1 M succinate-borate buffer of pH 6.8.[3] No increase in activity due to adenylic acid was observed. These experiments are summarized in Table III.

When the activity of the same enzyme preparation was compared in the two buffers, it was 15-fold higher in the succinate-borate buffer. This observation pointed to the possible inhibition of the enzyme by phosphate. Increasing quantities of phosphate were added to the system in succinate-

TABLE IV

Effect of Activators on Phosphate and Malate Inhibition

Specific enzyme activity, 14 μl. of CO_2 per microgram of N per 15 minutes. The data are average values of two similar experiments.

| Inhibitor | Concentration of activator | | Activity |
	MnSO₄	CoCl₂	
	M	M	$\mu l. CO_2$
None..............	0.00067		150
0.0067 M phosphate.. . .	0.00067		73
None		0.00017	183
0.0067 M phosphate..		0.00017	179
0.017 M malate...	0.00067		34
0.017 " "		0.00017	42

borate buffer. Fig. 3 shows the inhibiting effect of varying levels of phosphate. That succinate-borate is not stimulating was shown by the fact that its addition, at a level of 0.017 M, to a predominantly phosphate buffer system caused no increase in decarboxylating activity.

It seemed possible that the inhibition by phosphate was caused by a binding of the activator, manganese. Increasing quantities of manganous sulfate, up to 0.00335 M, were added to a system containing a constant small concentration of phosphate and a constant quantity of enzyme. No enhancement of activity resulted when the manganese level was increased.

It had been found in the meantime that Co^{++} activated the enzyme in lower concentrations than did Mn^{++}. When Co^{++} was used as an activator, phosphate was found not to inhibit the enzymatic decarboxylation of oxalacetate (Table IV).

Upon the addition of increasing amounts of Co^{++} to a system containing constant amounts of Mn^{++} and phosphate, a progressive reversal of inhi-

bition was found (Fig. 4). When these data were plotted in the manner customary for a competitive inhibition, a straight line was obtained (Fig. 5).

If the phosphate inhibition in the presence of Mn^{++} were merely caused by a binding of manganese by phosphate, one would have expected a com-
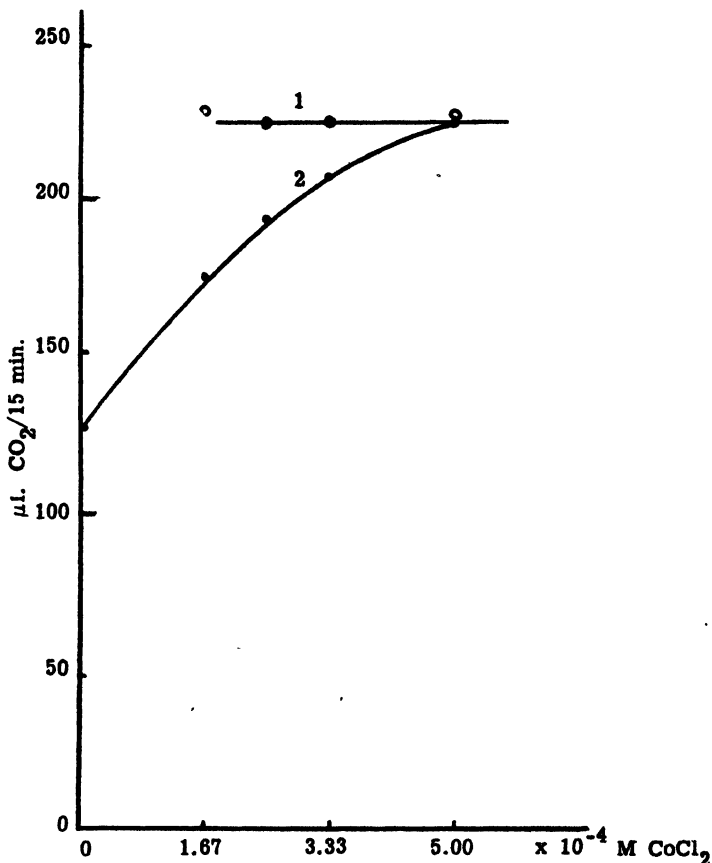
FIG. 4. The reversal of orthophosphate inhibition by Co^{++}. Curve 1, no orthophosphate or Mn^{++}; Curve 2, 0.0067 M orthophosphate and 0.00067 M Mn^{++}. Aqueous extract of bacterial acetone powder. The data are averages of two experiments.

plete reversal of inhibition upon the addition of a small yet maximally activating quantity of Co^{++}. The progressive nature of the reversal by increasing amounts of Co^{++} points to the formation of an inactive phosphate-manganese-enzyme complex. Apparently Co^{++} competes with the manganese-phosphate complex for the same place of attachment on the enzyme.

An attempt was made next to ascertain whether this inhibition was also of the substrate competitive type. Oxalacetate concentrations from 0.0017 M to 0.0303 M were employed in assays with a constant phosphate concentration (Table V). Although the data are variable, there is no consistent
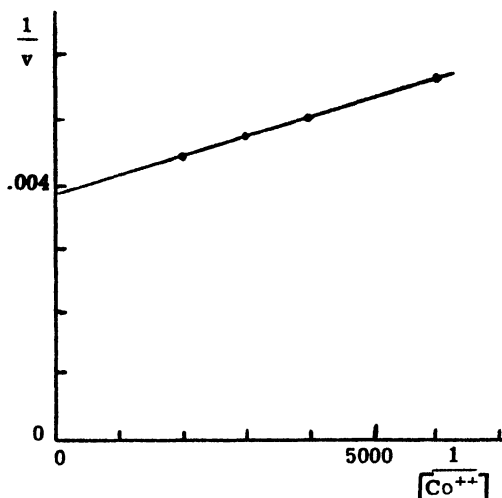
FIG. 5. The competitive reversal of orthophosphate inhibition by Co^{++}. The experiment is identical with that shown in Fig. 4. The reciprocal of the velocity, v, is plotted against the reciprocal of Co^{++} molarity. The velocity is expressed in microliters of CO_2 per 15 minutes.

TABLE V

Effect of Varying Concentrations of Oxalacetate upon Phosphate Inhibition

Specific activity of enzyme, 5.5 μl. of CO_2 per microgram of N per 15 minutes. The data are averages of six experiments.

Oxalacetate	Inhibition by 0.0067 M phosphate
	per cent
0.0017	46.0
0.0025	52.0
0.0034	55.4
0.0067	46.0
0.0101	40.5
0.0303	52.0

trend which would indicate a competitive type of inhibition. The enzymatic rate of decarboxylation was constant over the substrate concentration employed, while the chemical decomposition increased with rising oxalacetate. The latter is in keeping with observations of Straub (14) and

Ochoa *et al.* (22) that the chemical decarboxylation follows first order kinetics. Concentrations lower than 0.0017 M and higher than 0.0303 M oxalacetate were not employed, since the former provided too little gas for accurate measurement and in the latter case the large chemical evolution of carbon dioxide made it difficult to determine the enzymatic reaction with accuracy.

ATP was found to be about twice as active an inhibitor of the *Azotobacter* enzyme as the same molar concentration of orthophosphate (Fig. 3). Of the various phosphorus compounds tested, pyrophosphate was the most effective inhibitor of the enzyme (Fig. 3). · The inhibition by pyrophosphate is not the result of breakdown of this compound to orthophosphate, since the inhibition caused by the former is always more than twice as large as that caused by an equimolar concentration of orthophosphate. The enzyme does not exhibit apyrase activity.

TABLE VI

Increasing Concentrations of Oxalacetate versus Malate Inhibition

Specific enzyme activity, 7 μl. of CO_2 per microgram of N per 15 minutes. The data are averages of two experiments.

Oxalacetate	Inhibition by 0.0101 M malate
M	*per cent*
0.0034	53.9
0.0067	41.0
0.0134	56.8
0.0268	54.5

Lwoff *et al.* (23, 24) observed that the oxalacetate decarboxylase activity of resting cell suspensions of *Moraxella lwoffi* is inhibited by malate. Ochoa and Weisz-Tabori (25) found malate to inhibit the decarboxylase of *Micrococcus lysodeikticus* and of pigeon liver. Malate retarded the activity of the enzyme from *Azotobacter* (Fig. 3). The inhibition could not be competitively reversed by the addition of increasing quantities of substrate (Table VI). The extent of inhibition was the same in the presence of Mn^{++} or Co^{++} (Table IV).

Evans *et al.* (3) reported the complete inhibition of pigeon liver oxalacetate decarboxylase by 0.01 M malonate. The *Azotobacter* enzyme was not inhibited by this concentration of malonate.

Carbon Dioxide Fixation

Exchange reactions of $NaHC^{14}O_3$ and oxalacetate in the presence of the *Azotobacter* enzyme were carried out according to the technique described by Krampitz *et al.* (2).

In all cases the amount of carbon dioxide fixed in oxalacetate was exceedingly small; in no case did it constitute more than 0.113 per cent of the β-carboxyl carbon of the remaining oxalacetate. The fixation was not increased by 0.004 M ATP, 0.0067 M phosphate, 0.0067 M pyruvate, or 0.00067 M fumarate, either alone or in combination. The degree of fixation of the crude bacterial extract (specific activity, 1 μl. of CO_2 per microgram of N) was of the same order of magnitude as that of a purified preparation (specific activity, 80 μl. of CO_2 per microgram of N).

TABLE VII

Biotin Content versus Enzymatic Activity (Fractionation 32)

Fraction	Description of sample	Specific enzyme activity	Untreated hydrolysate	Phosgene-treated hydrolysate
		μl. CO_2 per γ N	mμgm. biotin per 100 μl. CO_2	mμgm. biotin per 100 μl. CO_2
A	Crude bacterial extract	0.5	1.94	
B	Acid-treated bacterial extract	4.0	1.35	1.51
D	Phosphate eluate of copper hydroxide	8.2	1.05	
F	Residue from 2.4 M ammonium sulfate	3.7	8.76	8.07
G	Residue from 3.4 M ammonium sulfate	14.0	0.79	1.03
I	Residue from 2.4 M ammonium sulfate (refractionation of Fraction G)	3.6	2.34	2.32
J	Residue from 3.4 M ammonium sulfate (refractionation of Fraction G)	35.3	0.73	0.78

Enzyme Activity and Biotin Content

The biotin content of some of the samples obtained during the fractionation of the *Azotobacter* enzyme was determined. *Lactobacillus arabinosus* was used as the test organism according to the method of Wright (26). The fractions were hydrolyzed by autoclaving at 15 pounds pressure for 2 hours in 2 N sulfuric acid to liberate bound biotin from the protein.

The results obtained (Table VII) seem to indicate that there was no increase in biotin concentration with respect to activity as a result of an increase in enzyme purity. It was found that the purest fraction described in Table VII, 32J, contains 0.017 mole of biotin per 100,000 gm. of protein (assuming a nitrogen content of 16 per cent). There was no increase in biotin activity upon treatment of hydrolysates with phosgene under the conditions of the Schotten-Baumann reaction, indicating that the samples contained no biotin diaminocarboxylic acid (Table VII). Control experi-

ments with pure biotin diaminocarboxylic acid[4] resulted in almost quantitative conversion to biotin following treatment with phosgene.

DISCUSSION

In initial studies on the purification of this enzyme it was found that fractional precipitation with solvents or salts of the original or acid-treated aqueous bacterial extract did not yield fractions of increased purity. This behavior suggested that the enzyme may be attached to some inert material. Since the extracts gave a positive Molisch test, it seemed possible that the enzyme might be associated with a polysaccharide. Herriott (27), working on the purification of swine pepsinogen, removed the enzyme protein from accompanying carbohydrates by adsorption on copper hydroxide gel and by elution with phosphate buffer. When this method was applied to the decarboxylase, the eluate still gave a positive Molisch test but it was now possible to increase appreciably the purity of the enzyme in the eluate by salt fractionation. The purified decarboxylase preparation from *Azotobacter* which has an activity of 40 μl. of CO_2 per 15 minutes per microgram of N is approximately 30 times as active as the purest fraction obtained from *Micrococcus lysodeikticus* (28) and 83 times more active than the pigeon liver preparation (20).

The competitive reversal by Co^{++} of the inhibition caused by phosphate in the presence of Mn^{++} indicates the existence of an inactive phosphate-manganese-decarboxylase complex. It seems possible that the Co^{++} competes with the phosphate-manganese complex for the same point of attachment on the enzyme. These observations are reminiscent of the inhibition of enolase by a magnesium-fluorophosphate complex (29). A similar inhibition of phosphoglucomutase has recently been reported by Najjar (30) in which the inhibitory agent is a complex of magnesium-fluoro-glucose-1-phosphate.

The apparent lack of carbon dioxide fixation into oxalacetate by the *Azotobacter* enzyme may be due to the fact that it only catalyzes the reaction

$$\text{Oxalacetic acid} \rightleftharpoons \text{pyruvic acid} + CO_2$$
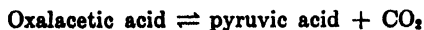
The equilibrium of this reaction is so far in the direction of decarboxylation ($K = 4.9 \times 10^3$ (3)) that it is impossible to demonstrate the exchange of carbon dioxide. The demonstrated ability of extracts of *Micrococcus lysodeikticus* and pigeon liver to incorporate carbon dioxide into oxalacetate must be ascribed to the presence of coupling enzymes in these preparations. Even the purified pigeon liver enzyme of Vennesland *et al.* (20) is probably

still an enzyme system rather than a single carboxylase. In this connection it is of interest that Ochoa (22) has proposed that the "malic" enzyme is either a single enzyme or a "functional enzyme unit."

It is tempting to speculate that oxalacetate decarboxylase may act as the common gateway enzyme for the fixation of carbon dioxide into dicarboxylic acids in conjunction with different coupling enzymes, such as transaminase, citrogenase, etc. The coupling reaction may then supply the necessary energy for the endergonic fixation of CO_2 with pyruvate (9).

SUMMARY

1. The oxalacetate decarboxylase of *Azotobacter vinelandii* has been purified 40- to 80-fold from aqueous extracts of bacterial acetone powder.

2. The enzyme was activated by Mn^{++}, Co^{++}, Zn^{++}, and slightly by Mg^{++}. Orthophosphate, pyrophosphate, and ATP were shown to inhibit the enzyme in the presence of Mn^{++}. Malate inhibited when Mn^{++} or Co^{++} was used as activator. Orthophosphate failed to slow the rate of enzymatic catalysis when Co^{++} was used for activation. The inhibition by orthophosphate in the presence of Mn^{++} was competitively reversed by the addition of increasing quantities of Co^{++}.

3. Fixation of carbon dioxide in appreciable quantities into oxalacetate could not be shown with the *Azotobacter* enzyme.

4. There was no increase in biotin concentration with respect to activity as a result of an increase in enzymatic purity. The most highly purified enzyme fraction which was assayed microbiologically for biotin activity was found to contain 0.017 mole of biotin per 100,000 gm. of protein.

BIBLIOGRAPHY

1. Krampitz, L. O., and Werkman, C. H., *Biochem. J.*, **35**, 595 (1941).
2. Krampitz, L. O., Wood, H. G., and Werkman, C. H., *J. Biol. Chem.*, **147**, 243 (1943).
3. Evans, E. A., Jr., Vennesland, B., and Slotin, L., *J. Biol. Chem.*, **147**, 771 (1943).
4. Wood, H. G., *Physiol. Rev.*, **26**, 198 (1946).
5. Lardy, H. A., Potter, R. L., and Elvehjem, C. A., *J. Biol. Chem.*, **169**, 451 (1947).
6. Shive, W., and Rogers, L. L., *J. Biol. Chem.*, **169**, 453 (1947).
7. Lichstein, H. C., and Umbreit, W. W., *J. Biol. Chem.*, **170**, 329 (1947).
8. Ochoa, S., Mehler, A., Blanchard, M. L., Jukes, T. H., Hoffmann, C. E., and Regan, M., *J. Biol. Chem.*, **170**, 413 (1947).
9. Lardy, H. A., Potter, R. L., and Burris, R. H., *J. Biol. Chem.*, **179**, 721 (1949).

10. MacLeod, P. R., and Lardy, H. A., *J. Biol. Chem.*, **179**, 733 (1949).
11. Korkes, S., and Ochoa, S., *J. Biol. Chem.*, **176**, 463 (1948).
12. Lee, S. B., Burris, R. H., and Wilson, P. W., *Proc. Soc. Exp. Biol. and Med.*, **50**, 96 (1942).
13. Lee, S. B., and Burris, R. H., *Ind. and Eng. Chem.*, **35**, 354 (1943).
14. Straub, F. B., *Z. physiol. Chem.*, **245**, 140 (1936).
15. Mayer, P., *Biochem. Z.*, **62**, 462 (1914).
16. Wieland, H., *Ann. Chem.*, **436**, 231 (1924).
17. Banga, I., and Szent-Györgyi, A., *Z. physiol. Chem.*, **245**, 113 (1936).
18. Breusch, F. L., *Biochem. J.*, **33**, 1757 (1939).
19. Vennesland, B., and Felsher, R. Z., *Arch. Biochem.*, **11**, 279 (1946).
20. Vennesland, B., Evans, E. A., Jr., and Altman, K. I., *J. Biol. Chem.*, **171**, 675 (1947).
21. Utter, M. F., and Wood, H. G., *J. Biol. Chem.*, **160**, 375 (1945).
22. Ochoa, S., Mehler, A. H., and Kornberg, A., *J. Biol. Chem.*, **174**, 979 (1948).
23. Lwoff, A., Andureau, A., and Cailleau, R., *Compt. rend. Acad.*, **224**, 303 (1947).
24. Lwoff, A., and Cailleau, R., *Compt. rend. Acad.*, **224**, 678 (1947).
25. Ochoa, S., and Weisz-Tabori, E., *J. Biol. Chem.*, **174**, 123 (1948).
26. Wright, L. D., *Biol. Symposia*, **12**, 290 (1947).
27. Herriott, R. M., *J. Gen. Physiol.*, **21**, 501 (1938).
28. Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S., *J. Biol. Chem.*, **174**, 961 (1948).
29. Warburg, O., and Christian, W., *Biochem. Z.*, **310**, 384 (1942).
30. Najjar, V. A., *J. Biol. Chem.*, **175**, 281 (1948).

AMINO ACIDS

VI. THE OXIDATION OF SALTS OF β-AMINO ALCOHOLS*

By JOHN H. BILLMAN, EARL E. PARKER,† AND WALTER T. SMITH‡

(From the Department of Chemistry, Indiana University, Bloomington)

In an earlier series of publications (1), it was shown that α-amino acids can be made from the corresponding β-amino alcohols by first protecting the amino group of the alcohol with an acyl radical, before oxidizing the carbinol group, and then removing the acyl radical after the formation of the carboxyl group.

A new method has been developed for producing α-amino acids from β-amino alcohols. This method consists in protecting the amino group of the β-amino alcohol by converting it into a substituted ammonium ion before oxidizing the carbinol group to a carboxyl group. The substituted ammonium ions are prepared by adding an acid such as sulfuric acid to the β-amino alcohols. The following series of reactions outlines the steps involved in the synthesis

$$\underset{\underset{\textstyle R-CH-CH_2OH}{|}}{NH_2} + H_2SO_4 \rightarrow \underset{\underset{\textstyle R-CH-CH_2OH}{|}}{NH_3^+SO_4H^-} \xrightarrow{[O]}$$

$$\underset{\underset{\textstyle R-CH-COOH}{|}}{NH_3^+SO_4H^-} \rightarrow \underset{\underset{\textstyle R-CH-C-OH}{| \quad \|}}{NH_2 \quad O}$$
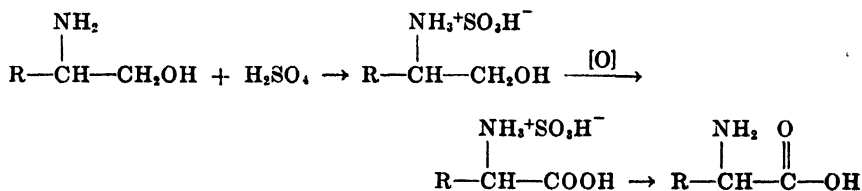
The four commercially available β-amino alcohols, 2-aminoethanol, 2-aminopropanol-1, 2-aminobutanol-1, and 2-methyl-2-aminopropanol-1, were each converted to the corresponding amino acid by the above procedure. In all cases, the yields were somewhat below those obtained by the acylation method (1). However, this new method has some advantages over the former one, in that fewer steps are involved, and it is less time-consuming.

The most suitable oxidizing agent was found to be potassium permanganate in an acidic medium.

Glycine—To a solution containing 72.5 gm. of concentrated sulfuric acid in 600 ml. of water were added, with vigorous stirring, 18.3 gm. of pure 2-amino ethanol. Then, while the stirring was continued, 63.2 gm. of potassium permanganate were added at such a rate that the temperature of the mixture remained below 50°. About 1 hour was required to add all of the permanganate.

The manganese dioxide, which formed during the oxidation, was removed by filtration and the filtrate was heated to boiling, and treated with a hot solution containing 170 gm. of barium chloride dihydrate dissolved in 500 ml. of water. This mixture was digested for several hours and filtered. The filtrate was evaporated to dryness under reduced pressure, and two 20 ml. portions of water were added to the residue and removed each time under reduced pressure. The process was repeated with three 20 ml. portions of absolute alcohol. The residue was extracted with 400 ml. of boiling 95 per cent alcohol and then with two more 100 ml. portions of boiling alcohol. Each extract was kept separate, cooled to room temperature, and filtered. 25 ml. of pyridine were added to the 400 ml. extract and 5 ml. were added to each of the other two extracts. The solutions were placed in a refrigerator for 24 hours when the glycine was filtered off and washed free from pyridine with absolute alcohol. A portion of the product obtained was oily. This part was recrystallized by dissolving it in water and adding 10 volumes of methyl alcohol. The total yield was 10 gm. (44.5 per cent) of pure sulfate- and chloride-free glycine.

$C_2H_5O_2N$.　Calculated, N 18.67; found, 18.88

Alanine, α-Amino-n-butyric Acid, and α-Aminoisobutyric Acid—These amino acids were prepared by the following procedure, which is similar to that used for making glycine.

To 0.3 mole of β-amino alcohol dissolved in 600 ml. of water containing 46 gm. of concentrated sulfuric acid were slowly added 63 gm. of potassium permanganate, the temperature being kept below 30°. About 2 hours were required. The manganese dioxide was removed and the filtrate was treated with 110 gm. of barium chloride dihydrate dissolved in 500 ml. of water. After removing the barium sulfate, evaporating, and drying the residue as already described, the amino acid was extracted with two 100 ml. portions of boiling 95 per cent alcohol. The extracts were combined, cooled to room temperature, and filtered. To the filtrate were added 30 ml. of freshly distilled aniline. The mixture was placed in a refrigerator for 24 hours, after which the amino acid crystals were filtered off and washed with several small portions of absolute alcohol. The yield of crude alanine was 12.2 gm. (48 per cent), and of crude α-amino-n-butyric acid 11 gm.

(36 per cent). The impure products were recrystallized by dissolving them in a small amount of hot water, filtering, and adding several volumes of methyl alcohol. The yields of pure alanine and of pure α-amino-n-butyric acid were 10 gm. (35 per cent) and 9 gm. (29 per cent) respectively. A yield of 15 gm. (48.4 per cent) of pure α-aminoisobutyric acid was obtained without recrystallization.

Amino acid prepared	Per cent N calculated	Per cent N found
α-Aminopropionic acid..	15.73	15.47
α-Amino-n-butyric "	13.59	13.71
α-Aminoisobutyric "	13.59	13.47

The phthalyl derivatives (2) as well as the N-2,4-dinitrophenyl derivative of all of the above amino acids were prepared and found to have melting points identical to the derivatives prepared from amino acids made by other methods.

SUMMARY

A new method has been developed for the preparation of α-amino acids from β-amino alcohols. Four α-amino acids have been prepared by oxidizing the salts of the corresponding β-amino alcohols.

BIBLIOGRAPHY

1. Billman, J. H., and Parker, E. E., J. Am. Chem. Soc., 66, 538 (1944).
2. Billman, J. H., and Harting, W. F., J. Am. Chem. Soc., 70, 1473 (1948).

THE SEDIMENTATION BEHAVIOR AND MOLECULAR WEIGHT OF PANCREATIC CARBOXYPEPTIDASE*

BY EMIL L. SMITH, DOUGLAS M. BROWN, AND H. THEO HANSON†

(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City)

Previous studies of the physical properties of crystalline carboxypeptidase have included investigations of the electrophoretic behavior (1, 2) and measurements of viscosity and diffusion (2). It was reported that the several times recrystallized protein migrated essentially as a homogeneous substance in the electrophoresis apparatus (2).

We have investigated the sedimentation behavior of carboxypeptidase in the ultracentrifuge, since no previous studies of this type have been reported. It was found that the carefully recrystallized protein behaved as a homogeneous substance. From the sedimentation constant and the previously determined diffusion constant (2), the molecular weight was found to be 33,800. This value is in reasonable agreement with the molecular weight of 31,600 estimated from diffusion and viscosity measurements by Putnam and Neurath (2).

EXPERIMENTAL

The enzyme was prepared from frozen bovine pancreas as described by Anson (3) and recrystallized four to six times by the procedure of Neurath, Elkins, and Kaufman (4). The protein concentration of the carboxypeptidase solutions was evaluated from the protein N content, the conversion factor of 7.0 being used, since the protein has been reported to contain 14.4 per cent N (3).

All of the sedimentation studies were made in the Spinco[1] electrically driven ultracentrifuge. A brief description of this instrument, and the controls and procedures, has been given in a previous publication from this laboratory (5).

The results obtained with various preparations of carboxypeptidase are presented in Table I. Most of the runs showed that the protein sedimented essentially as a homogeneous substance with a sedimentation

[1] Specialized Instruments Corporation, Belmont, California.

constant of about 3.1×10^{-13}. A representative experiment is shown in Fig. 1, A. However, in some experiments it was found that a frequent

<div align="center">

TABLE I

Sedimentation of Carboxypeptidase

</div>

The temperature is the average for the duration of the run. The results incorporate the usual corrections for the temperature, and for the viscosity and density of the solvent (6). The values for the sedimentation constants (S_1, S_2, and S_3) are for $S_{20,w}$ in cm. per second. The parenthetical values give the amount of each component estimated from its percentage of the total sedimenting area.

Sample No.	Solvent	pH	Temperature	Protein concentration	$S_1 \times 10^{13}$	$S_2 \times 10^{13}$	$S_3 \times 10^{13}$
			°C.	per cent			
1	0.04 M phosphate + 0 4 M NaCl	7.1	25.0	0.42	3.25		
2	0.015 M veronal + 0.185 M NaCl	7.7	24.8	0.23	3 19		
3	0.015 M veronal + 0.185 M NaCl	7.7	27.1	0.12	3.29		
4	0.015 M veronal + 0.185 M NaCl	7.7	22.2	0.06	3.02		
5	0.015 M veronal + 0.185 M NaCl	7.7	25.3	0.23	3.45*		
6	0.25 M NaCl	5.9	24.4	0.12	3.12		
7	0.1 " veronal + 0.1 M NaCl	7.8	26.2	0.14	2.97		
8	0.1 M veronal + 0.3 M NaCl	7.8	26.4	0.16	2.83		
9†	1.0 M NaCl	6.8	24.7	0.88	2.82		
10	1.0 " "	6.5	25.1	0.42	2.94		
11†	0.015 M veronal + 0.385 M NaCl	7.6	25.3	0.91	3.02 (86%)	9.0 (14%)	
12	1.0 M NaCl	5.9	21.8	0.50	3.05 (50%)	8.2 (8%)	3.9 (42%)
13	0.5 " "	5.9	23.9	0.25	3.32 (65%)	9.7 (8%)	5.8 (27%)
14	0.04 M glycine + 0.11 M NaCl	9.0	24.5	0.44	3.15 (87%)	9.4 (13%)	
15	0.2 M phosphate	7.6	22.2	0.28	2.88 (90%)	8.6 (10%)	
16	0.2 " NaCl	8.7	22.0	0.26	3.26 (87%)	9.1 (13%)	
17	0.1 " glycine + 0.1 M NaCl	11.4	22.3	0.89	2.02*		

* These values have been omitted from the average sedimentation constant.

† These are the experiments shown in Fig. 1, A (Sample 9) and Fig. 1, B (Sample 11).

contaminant of carboxypeptidase is an accompanying protein with a sedimentation constant of about 9.0×10^{-13}; such a run is illustrated in Fig. 1, B. This heavier protein is apparently a globulin which is relatively in-

soluble at low ionic strengths, since extracts of a stock preparation of the crystalline enzyme made near neutrality at 0.2 ionic strength do not show this substance. When extracts are made at higher salt concentrations (0.4 to 1.0 ionic strength), or at about pH 9, this substance is dissolved. In two instances, large amounts of additional contaminants (S_3) were observed, as in Samples 12 and 13. These proteins, S_2 and S_3, do not appear to possess any carboxypeptidase activity. Enzymatic tests with homogeneous samples yielded values for the proteolytic coefficient (C)[2] of 12 to 13, with carbobenzoxyglycyl-L-phenylalanine as the substrate at 0.05 M concentration. Tests with Samples 12 and 13 gave somewhat lower activities (C = 7 to 9). Previous results with highly purified carboxy-
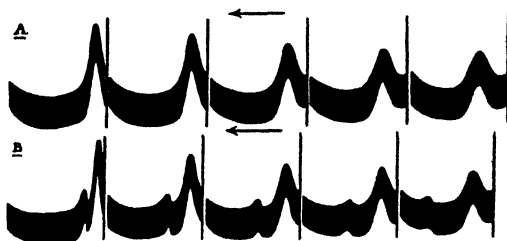
Fig. 1. The sedimentation behavior of carboxypeptidase in the ultracentrifuge. *A* shows the results with a homogeneous preparation (Sample 9) measured in 1.0 M sodium chloride. The first picture was taken 35 minutes after attaining full speed (59,780 R.P.M.); subsequent photographs were made at 16 minute intervals. *B* shows a preparation (Sample 11) which contains some inactive heavier material. The first exposure was made 16 minutes after reaching 59,780 R.P.M.; the intervals between the subsequent photographs were 16, 8, 8, and 16 minutes. The arrow indicates the direction of sedimentation from the meniscus.

peptidase gave proteolytic coefficients in the neighborhood of 12 to 14 (4, 7, 8).

We have taken advantage of the insolubility of the contaminating protein in 0.2 ionic strength sodium chloride in order to prepare routinely carboxypeptidase which behaves as a homogeneous substance in the ultracentrifuge, and which possesses a C value of about 12 to 13. Stock preparations of 4 times crystallized carboxypeptidase are extracted with 0.2 M sodium chloride and centrifuged. The extracts are then dialyzed in the usual manner against gradually decreasing concentrations of salt solutions and finally against distilled water. Preparations of carboxypeptidase twice recrystallized in this way are homogeneous even when extracted into 1.0 M sodium chloride (Samples 9 and 10).

[2] The proteolytic coefficient is defined as the first order velocity constant expressed in decimal logarithms for a solution containing 1 mg. of protein N per cc.

A single determination (Sample 17) performed at pH 11.4 gave a preparation which showed only a single boundary, but which had a sedimentation constant of about 2.0 × 10⁻¹³. This low value suggests that carboxypeptidase is split into half particles at this pH. It was found that this preparation was completely inactive enzymatically when tested at pH 7.4 in veronal buffer with carbobenzoxyglycyl-L-phenylalanine as the substrate.

The data in Table I indicate no significant variation of the sedimentation constant of carboxypeptidase (S_1) over the pH range of 5.9 to 9.0, over the tested range of protein concentrations from 0.06 to 0.91 per cent, or at different ionic strengths. Therefore, all of the values for S_1 were averaged with the exception of Sample 17, measured at pH 11.4, and Sample 5, which gave an unaccountably high result. The average value for $S_{20,w}$ is 3.07 × 10⁻¹³ with an average deviation of ±0.14 and a standard deviation of ±0.15.

The diffusion constant of carboxypeptidase has been reported by Putnam and Neurath (2) to be 9.94 × 10⁻⁷ sq. cm. per second at 25°. When this value is corrected for the difference in the viscosity of water between 25° and 20°, $D_{20,w}$ is 8.82 × 10⁻⁷. Assuming that V is 0.75, and incorporating these values in the usual formula (6), $M = RTS/D(1 - V\rho)$, we obtain 33,800 for the molecular weight.

SUMMARY

Crystalline bovine pancreatic carboxypeptidase sediments as a homogeneous substance in the ultracentrifuge. The average value for the sedimentation constant, $S_{20,w}$, is 3.07 ± 0.15 × 10⁻¹³. From this and diffusion data, the molecular weight is 33,800.

The crystalline enzyme frequently contains a contaminating globulin with $S_{20,w} = 9.0 × 10⁻¹³$. This inactive substance is insoluble at low ionic strengths near neutrality and can be separated from carboxypeptidase.

BIBLIOGRAPHY

1. Ågren, G., and Hammarsten, E., *J. Physiol.*, **90,** 330 (1937).
2. Putnam, F. W., and Neurath, H., *J. Biol. Chem.*, **166,** 603 (1946).
3. Anson, M. L., *J. Gen. Physiol.*, **20, 663** (1937).
4. Neurath, H., Elkins, E., and Kaufman, S., *J. Biol. Chem.*, **170,** 221 (1947).
5. Smith, E. L., Brown, D. M., Fishman, J. B., and Wilhelmi, A. E., *J. Biol. Chem.*, **177,** 305 (1949).
6. Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford (1940).
7. Stahmann, M. A., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **164, 753** (1946).
8. Smith, E. L., *J. Biol. Chem.*, **175,** 39 (1948).

THE METABOLISM OF THE ORGANIC ACIDS OF TOBACCO LEAVES

II. EFFECT OF CULTURE OF EXCISED LEAVES IN SOLUTIONS OF d-ISOCITRATE AND ACETATE

By HUBERT BRADFORD VICKERY and MARJORIE D. ABRAHAMS

(From the Biochemical Laboratory of the Connecticut Agricultural Experiment Station, New Haven)

It has recently been shown (1) that certain organic acids, when administered to excised tobacco leaves during culture experiments carried out in darkness, exert a marked influence upon the course of the metabolic reactions of the tissues. In control samples cultured on water, a considerable part of the malic acid present at the start disappears and additional citric acid is formed, the total organic acidity remaining essentially constant. However, if citrate, succinate, or fumarate, for example, is acquired from the culture solution, both malic and citric acids increase, and if malate is administered, the formation of citric acid is stimulated. Tobacco leaves obviously contain enzyme systems that provide for rapid and extensive metabolic transformations of the organic acids present or introduced into the cells, these transformations being especially noteworthy when the organic acid added from the culture solution is one of the members of the Krebs tricarboxylic acid cycle. On the other hand, little disturbance of the normal sequence of reactions was observed when the leaves were cultured on tartrate, an acid which is not naturally present in tobacco leaves and which is not a member of the Krebs cycle.

A further series of experiments is described in the present paper. These were carried out in order to extend the observations to include d-isocitric acid. A different variety of tobacco was employed, and tests were accordingly made to see whether the effect of added citrate or malate was similar to that observed earlier. In addition, the effect of added acetate has been examined.

EXPERIMENTAL

Tobacco plants (*Nicotiana tabacum*) of the variety known as "Connecticut shade-grown" were grown in sand in the greenhouse, a culture solution that provided all of the nitrogen as nitrate being employed. The samples were collected 55 days after the seedlings had been transplanted and at the time that the first flowers were opening. A recently developed sampling technique based upon a systematized Latin square was employed (2),

according to which twelve leaves were cut from each of twelve plants and distributed to six samples in such a way that each plant and each leaf position was equally represented in each sample. This method has been shown to be advantageous in diminishing the statistical variation in composition among presumably identical samples of leaves.

As in the previous work, the culture solutions used for the treatment of the leaves were 0.2 M in concentration. The solution of potassium L-malate (pH 6.5) was prepared from purified diethyl L-malate of $[\alpha]_D^{24} = -10.7°$ isolated from tobacco leaves by the late Dr. G. W. Pucher of this laboratory in the course of earlier work. The potassium d-isocitrate (pH 6.8) was prepared from l-dimethyl isocitrate lactone obtained from *Bryophyllum* leaves. The citrate (pH 5.9) and acetate (pH 6.8) were prepared from reagent grade acids. The technique of the leaf culture experiments was the same as in the earlier work. The culture period was 48 hours.

With the exception of the leaves cultured on acetate, all of the samples increased slightly in fresh weight and maintained full turgor. There was evidence of a minor degree of chlorophyll degeneration, the leaves, although fully green, being faintly mottled, especially along the main veins. The leaves cultured on acetate became completely flaccid within 24 hours and at the end were unusually thin and fragile, having lost about 43 per cent of their fresh weight, mainly through the evaporation of water.

The analytical methods for total acidity, oxalic, malic, and citric acids were those long in use in this laboratory (3). Acetic acid was determined by distillation with steam under controlled conditions. Isocitric acid was determined by a method in which heart muscle aconitase is employed essentially as described by Krebs and Eggleston (4). The equilibrium mixture of citric, isocitric, and *cis*-aconitic acids that is formed under the influence of aconitase has been found to contain close to 89 per cent of citric acid[1] in agreement with the observations of Krebs and Eggleston.

The results of the analysis of the dried samples are shown in Table I. Inasmuch as the total nitrogen content is not affected by the treatment, the nitrogen determinations serve to illustrate the degree of variation among the samples. The mean nitrogen content of the six samples was 4.94 ± 0.14 gm. per kilo of initial fresh weight. The coefficient of variation was 2.9 per cent, a part of which represents the analytical as distinct from the sampling error (2). The coefficient of variation of the fresh weight was 2 per cent.

Culture on Water—The organic solids decreased by 8 per cent, an evidence of a relatively high rate of respiratory activity in this lot of leaves. The ash weight was essentially unchanged and the apparent increase of

[1] Unpublished observations of Dr. C. A. Hargreaves of this laboratory.

total organic acids of 9.7 m.eq. per kilo is also probably negligible, being only 2.7 per cent of the total acidity. It should be pointed out that the total organic acidity of this variety of tobacco is substantially greater than that of the kinds used in the experiments of the previous paper, but the present data are not out of line with earlier observations on shade-grown tobacco (5).

During the culture period, the total organic acids, calculated arbitrarily as citric acid, increased from 23.6 to 26.4 per cent of the organic solids. The change computed in these terms was, however, apparent only, being almost entirely due to the loss of solids through respiration.

TABLE I

Effect of Culture on 0.2 M Potassium Salts of Organic Acids upon Composition of Excised Tobacco Leaves

The figures not otherwise designated represent milliequivalents per kilo of original fresh weight of leaves.

	Control before culture	Changes in 48 hrs. of culture in darkness				
		Water	Citrate	L-Malate	d-Iso-citrate	Acetate
Total nitrogen, gm..............	5.07	−0.13	−0.17	+0.06	−0.31	−0.25
Organic solids, "	98.8	−7.9	−6.8	−6.7	−6.2	−3.2
Inorganic solids, "	20.5	−0.5	+15.9	+14.6	+12.7	+13.2
" " as potassium carbonate.....................			+230	+212	+183	+190
Total organic acids..............	364	+9.7	+196	+170	+173	+131
Oxalic acid.............	26.8	+1.4	+0.8	+1.1	−0.6	+1.3
Citric "	43.1	+49.5	+151	+159	+80.0	+21.6
Malic "	215	−55.7	+27.2	−33.0	−11.7	−31.0
Isocitric acid.............					+78.6	
Acetic acid.........						+114
Undetermined acid.........	79.9	+14.5	+16.9	+43.4	+26.6	+25.7
pH of dry tissue at end..........	5.62	+0.17	+0.33	+0.44	+0.30	+0.89

As in the previous work, there was a marked increase in citric acid; 49.5 m.eq. per kilo of newly formed citric acid appeared, an increase of 115 per cent over the quantity present at the start. This was accompanied by a decrease in malic acid of 55.7 m.eq. or 26 per cent of the amount originally present. The increase in undetermined acids was only 14.5 m.eq. and, inasmuch as this quantity is computed by difference, was probably not far from the limit of significance; in any case, the production of undetermined acids was small. There was no significant effect upon oxalic acid. The general behavior of this sample therefore conformed closely with earlier experience with this and other varieties of tobacco.

Culture on Citrate—The total organic acids of the leaves increased by

196 m.eq. per kilo of initial fresh weight. This is equivalent to an uptake of 12.5 gm. of citric acid or 13.6 per cent of the organic solids present at the end of the treatment. The quantity calculated from the increase in ash, assuming this increase to represent potassium carbonate, was 14.8 gm. The leaves at the end were thus enormously enriched in organic acids; the 560 m.eq. ultimately present, calculated arbitrarily as citric acid, amount to 35.8 gm. per kilo or 39 per cent of the organic solids of the tissues.

In spite of the gain of more than 12 gm. of citric acid, the leaves lost 6.8 gm. of organic solids during the 48 hour period. Respiration must therefore have been greatly stimulated as compared with the control sample cultured on water.

Culture on L-*Malate*—The present experiment differs from that of the previous study in that L-malate was used rather than DL-malate. Accordingly, the results are not obscured by the probable failure of D-malate to share in the enzymatic reactions. The total organic acids increased by 170 m.eq., the equivalent of 11.4 gm. of malic acid if this figure is assumed to represent the uptake of malate ion. The figure calculated from the increase in ash is 14.2 gm. The loss of organic solids during the culture period was 6.7 gm. and the evidence therefore again points to a greatly stimulated respiration under the experimental conditions. If the total acidity of the tissues at the end is arbitrarily computed as citric acid, the leaves contained 37 per cent of their organic solids as organic acids, thus showing enrichment comparable with that obtained in the culture experiment with citrate.

Culture on d-*Isocitrate*—The total organic acids increased by 173 m.eq., indicating that 11 gm. of isocitric acid were taken up. Calculation from the increase in ash gives 11.7 gm. The loss of organic solids was 6.2 gm., so that it would appear that about 17 gm. of organic substance were respired by the leaves in the course of the experiment, again an evidence of stimulation and an indication that isocitric acid enters readily into the enzymatic reactions of the cells. The tissues at the end contained 37 per cent of the organic solids as organic acids on the same assumptions as before.

Culture on Acetate—The total organic acids increased by 131 m.eq., indicating that at least 7.9 gm. of acetic acid were taken up. The increase in ash was equivalent to an uptake of 11.4 gm. The loss of organic solids was 3.2 gm., so that the total respiration amounted to 11 gm. or more of organic material. Thus, in spite of the flaccidity of the leaves during most of the culture period, respiration was stimulated in comparison with the control sample cultured on water. As in the other samples, the leaves became enriched in organic acids; on the same assumptions as before, the

tissues contained close to 33 per cent of organic acids at the end of the culture period.

<div style="text-align:center">DISCUSSION</div>

The use of a method based on statistical principles for collecting the present samples makes it feasible to consider, at least in a preliminary way, the molar ratios between the main organic acid reactants in the system. It may be tentatively assumed that, with the gradual influx of organic acid anions from the culture solution, a drifting series of equilibrium conditions is set up. The change in composition at the end of the arbitrarily selected period of 48 hours permits estimates to be made of the relative quantities of organic acids metabolized during this period.

In the control leaves cultured on water, 49.5 m.eq. (16.5 mM) of citric acid appeared and 55.7 m.eq. (27.8 mM) of malic acid disappeared. The molar ratio of malic to citric acid is therefore 1.7, which suggests that, within reasonable limits of error, 2 moles of malic acid underwent transformations that resulted in the production of 1 mole of citric acid.

In the experiments with culture solutions, there are two methods available to calculate the total quantity of organic acid that entered the tissues. One estimate may be derived from the increase in ash. If the potassium salt was taken up as such by the open ends of the leaf vessels and migrated to the cells, then potassium ion equivalent to the total uptake of anion must remain in the tissue regardless of the ultimate fate of the anion. The increase in ash, expressed as milliequivalents of potassium carbonate, is therefore a measure of this uptake of acid, provided that the culture solutions were at such a pH that the acid they contained was fully neutralized.[2] The other estimate of the quantity of acid acquired by the sample is derived from the direct determination of the increase in total organic acidity. These two estimates have previously been shown to be closely correlated; however, they rarely agree with each other, the first being almost invariably somewhat larger. The difference between them can be assumed to represent that part of the newly acquired acid which underwent complete oxidation or, at least, oxidation to non-acidic products of metabolism.

In the leaves cultured on citrate, citric acid increased by 151 m.eq. If it is assumed, from the increase in ash, that 230 m.eq. of citrate were taken up (Table I), the difference, 79 m.eq. (26.3 mM), represents the citric acid metabolized. The increase in malic acid was 27.2 m.eq. (13.6 mM), and

---

[2] The L-malate at pH 6.5 and the isocitrate at pH 6.8 contained more than 95 per cent of the theoretical quantity of potassium ion; the citrate at pH 5.9 contained roughly 73 per cent of trivalent and 23 per cent of divalent citrate ion. Accordingly, the calculation of acid uptake from ash increase probably furnishes a slight underestimate.

the molar ratio of malic acid produced to citric acid consumed was therefore 0.52. This calculation neglects the apparent increase in undetermined acids. If the figure 16.9 m.eq. is assumed to be an accurate measure of this quantity, and these substances are, further, assumed to be derived from citric acid, the molar ratio becomes 0.66. Either calculation assumes that 34 m.eq. or 2.17 gm. of citric acid were completely metabolized.

If, on the other hand, the increase of 196 m.eq. of total acidity is taken as the measure of the uptake of citric acid, only 45 m.eq. of citric acid were metabolized with the production of malic acid. Neglecting the increase in undetermined acids, the molar ratio is 0.91; including it, the ratio becomes 1.5. The data as a whole suggest that from 1 to 2 moles of citric acid were involved in the production of 1 mole of malic acid. Regardless of the uncertainty of the analytical determinations, the evidence is conclusive that when the tissues are, as it were, flooded with citric acid the normal malic to citric transformation is reversed and the present results are thus in agreement with the earlier work.

The leaves cultured on L-malate took up 212 m.eq. of malate as computed from the increase in ash. Nevertheless, the malic acid content of the leaves decreased by 33 m.eq., so that it may be assumed that 245 m.eq. (123 mM) of malic acid were metabolized during the culture period. Citric acid increased by 159 m.eq. (53 mM); accordingly the molar ratio of the malic acid converted to citric acid was 2.3. If the apparent increase in undetermined acids of 43 m.eq. is assumed to be accurate and is applied as a correction, only 202 m.eq. of malic acid were converted to citric acid, giving a molar ratio of 1.9. This calculation assumes that 39 m.eq. or 2.6 gm. of malic acid were completely oxidized.

A similar calculation on the assumption that the increase in total acidity of 170 m.eq. represents the uptake of malate also gives a molar ratio of 1.9; if the correction for the increase in undetermined acids is applied, this ratio becomes 1.5. The evidence, therefore, rather strongly suggests, as in the case of culture on water, that reactions occurred in which 2 moles of malic acid were consumed in the formation of 1 mole of citric acid, a conclusion that is not unreasonable on theoretical grounds (1).

The leaves cultured on d-isocitrate took up 183 m.eq. of isocitric acid as computed from the increase in ash and contained 78.6 m.eq. of this substance at the end of the culture period. Thus 104.4 m.eq. of isocitric acid were presumably metabolized. Citric acid increased by 80 m.eq. and, if a mole for mole transformation of isocitric to citric acid is assumed to have occurred, there remain 24.4 m.eq. of isocitric acid available for other transformations. In addition, malic acid decreased by 11.7 m.eq., so that the total acid available for metabolism was 36 m.eq. The undetermined acids increased by 26.6 m.eq. and there is thus a balance of about 10 m.eq.

of acid unaccounted for. The estimate of isocitric acid completely oxidized computed from the difference between the increase in ash and the direct titration of acidity is also 10 m.eq.

If a similar calculation is based upon the increase of 173 m.eq. of organic acids in the tissues, only 14 m.eq. of isocitric acid were available for transformations other than to citric acid. Together with the decrease in malic acid, this approximately balances the increase in undetermined acids.

The leaves cultured on acetate took up 190 m.eq. of acetic acid, as calculated from the increase in ash of which 114 were found still present at the end; thus 76 m.eq. entered into the metabolism. It seems unlikely that the normal transformation of malic to citric acid was influenced in this case; citric acid increased by 21.6 m.eq. and malic acid decreased by 31 m.eq., the molar ratio being 2.2, a reasonable value in view of the results already mentioned. If it is assumed that the acetic acid took no direct share in this transformation, and if the increase of 25.7 m.eq. of undetermined acids represents a sufficiently accurate estimate of the fate of part of the acetic acid, there still remain 50 m.eq. of acetic acid to be accounted for. The estimate of acetic acid completely oxidized is 59 m.eq. and the agreement of these two last figures is perhaps as close as can be expected. That an appreciable part of the acetic acid was completely oxidized is obvious from the decrease in the acidity of the tissues from pH 5.6 to 6.5 in the course of the experiment.

If the calculation is based upon the increase in total organic acidity, which amounted to 131 m.eq., only 17 m.eq. of acetic acid appear to have been metabolized. This is insufficient to account for the increase in undetermined acids, although this is not certain because of the error in the measurement of the latter quantity. However, the view that so small a quantity of acetic acid as 17 m.eq. was metabolized is not consistent with the marked change in the pH of the tissues nor with the evidence, from the loss of organic solids, of a stimulated respiration. The results point to the estimate of acid absorbed from the increase in ash weight as giving the more likely general picture of the situation.

That the tobacco leaf can metabolize acetic acid has been demonstrated unequivocally by Krotkov and Barker (6), who allowed a leaf to absorb acetate marked with radioactive carbon in the carboxyl group for a short time and then studied the fate of the radioactivity during a subsequent period of respiration in darkness. After about 21 hours, a substantial fraction of the radioactivity absorbed by the leaf, and shown to be present in a non-volatile form at an earlier stage, was found in the respired carbon dioxide.

The demonstration that d-isocitrate is readily metabolized in the tobacco leaf is of considerable significance. It seemed highly probable from the

earlier work that this member of the Krebs cycle would share, with the other members that were tested, the property of entering into the metabolic reactions of the leaf. Experiment has shown that this expectation is realized in spite of the fact that the tobacco leaf does not normally contain demonstrable quantities of isocitric acid. Determinations were made in all of the samples. The calculation from the quantity of citric acid present after treatment of extracts with heart muscle aconitase, the possible presence of cis-aconitic acid in the solutions being neglected, gave small negative quantities (-4.9, -4.9, and -2.8 m.eq. per kilo respectively) in the control sample and in the samples cultured on water and on acetate, and small positive quantities (5.6 and 2.1 m.eq. per kilo) in the samples cultured on citrate and on malate. These results are of the order of 1 per cent or less of the total organic acidity of the samples and are well within the limits of error. However, after culture of the leaves on isocitrate, the presence of a substance that responded to the analytical method for isocitric acid was readily demonstrated.

The most important observation is that d-isocitric acid introduced into the vascular system of tobacco leaves appears to be readily converted into citric acid. Of the 80 m.eq. per kilo of newly formed citric acid, only a small part could have been derived from malic acid, for the decrease of this substance was only about 12 m.eq. The observation implies that there is an efficient enzyme system present, the function of which is analogous to that of the aconitase of mammalian heart muscle. That the intermediate substance is cis-aconitic acid remains to be demonstrated, although this seems highly probable in view of the experiments of Martius (7) and of Johnson (8) with the aconitase of cucumber seed extracts.

In none of the present culture experiments was there a significant effect upon the oxalic acid content of the leaves, notwithstanding the apparently greatly stimulated respiration in the leaves cultured on organic acids. The position of oxalic acid in the metabolic system of the tobacco leaf is still to be ascertained.

The available evidence suggests that tobacco leaves contain a complex of metabolic systems in which organic acids are concerned. The system in which malic and citric acids are mainly involved appears to have analogies with the Krebs tricarboxylic acid cycle, for each member of this cycle that has been studied exerts an influence upon the proportions of these two acids present at the end of a period of culture in darkness. Other systems are, however, present which may or may not be metabolically connected. Lactic acid would appear to have some metabolic connection since leaves cultured on lactate became enriched in both malic and citric acid (1); and there are good theoretical grounds (for a review see Ochoa (9)) for assuming such a connection to exist. Tartaric acid, however, does not

seem to fit into this system; leaves cultured on tartrate became enriched in organic acids but there was no obvious effects upon the malic or citric acid content. Whether the tartrate was itself metabolized to any significant extent was not ascertained.

Acetic acid also does not seem to have played a rôle in connection with the malic and citric acid metabolism; nevertheless, it was itself extensively metabolized. Its position, therefore, is similar neither to that of lactic acid nor, apparently, to that of tartrate.

SUMMARY

When leaves of tobacco (*Nicotiana tabacum*) of the variety "Connecticut shade-grown" are cultured for 48 hours on water in darkness, the malic acid content decreases and that of citric acid increases in a manner consistent with the view that 2 moles of malic acid undergo transformations that result in the formation of 1 mole of citric acid. This reaction is partially reversed when the leaves are cultured on citrate, from 1 to 2 moles of citric acid disappearing while 1 mole of malic acid appears.

When cultured on L-malic acid, the transformation of malic acid to citric acid is greatly stimulated and the behavior indicates that 2 moles of malic acid are required for the formation of 1 mole of citric acid.

d-Isocitric acid, although not normally present in the tobacco leaf in demonstrable quantities, enters readily into the metabolism of the leaves, the chief product being citric acid. Accordingly, an enzyme system capable of bringing about this transformation appears to be present.

Acetic acid also enters into the metabolism; although it has no apparent effect upon the normal transformation of malic to citric acid, a significant part of the acetate introduced disappears entirely, presumably being completely oxidized.

Culture of the leaves on organic acid salts in all cases greatly increased the respiration as measured by the loss of organic solids and resulted in tissues abnormally enriched in organic acids.

BIBLIOGRAPHY

1. Pucher, G. W., and Vickery, H. B., *J. Biol. Chem.*, **178**, 557 (1949).
2. Vickery, H. B., Leavenworth, C. S., and Bliss, C. I., *Plant Physiol.*, in press.
3. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., *Connecticut Agr. Exp. Sta., Bull. 496* (1946).
4. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **38**, 426 (1944).
5. Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., *Connecticut Agr. Exp. Sta., Bull. 399* (1937).
6. Krotkov, G., and Barker, H. A., *Am. J. Bot.*, **35**, 12 (1948).
7. Martius, C., *Z. physiol. Chem.*, **257**, 29 (1938).
8. Johnson, W. A., *Biochem. J.*, **33**, 1046 (1939).
9. Ochoa, S., in Green, D. E., Currents in biochemical research, New York, 165 (1946).

PARTIAL PURIFICATION OF ACONITASE

By JOHN M. BUCHANAN* AND CHRISTIAN B. ANFINSEN†

(From the Biochemical Division of the Medical Nobel Institute, Stockholm, Sweden)

In 1936, Martius and Knoop (1) discovered an enzyme which catalyzed the following reactions:

$$\text{Citric acid} \underset{-\,H_2O}{\overset{+\,H_2O}{\rightleftharpoons}} \textit{cis-}\text{aconitic acid} \underset{-\,H_2O}{\overset{+\,H_2O}{\rightleftharpoons}} \text{isocitric acid}$$
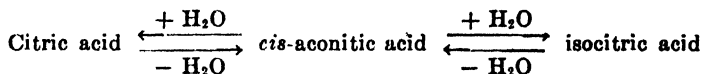
This enzyme was later named aconitase by Breusch (2). At equilibrium, the three substances, citric, aconitic, and isocitric acids, exist in the approximate molecular ratio of 89:3:8 (3). Jacobsohn and his associates (4, 5) have provided indirect evidence that "aconitase" may be composed of two separate enzymes, each specifically catalyzing a single reaction, but this point has never been definitely established.

Johnson has studied the kinetics of aconitase as well as its relative activity in different tissues (6). Krebs and Eggleston (3) have contributed further information on the properties of aconitase, including data on various stabilizers and inhibitors of the enzyme. Although Ochoa has concentrated the enzyme by ammonium sulfate precipitation (7), he has not attempted to fractionate or purify it. Contrary to previous reports, aconitase may be precipitated from solution by alcohol, and even acetone, without loss of activity, provided the temperature is kept sufficiently low. Alcohol was used as a fractionating agent in the purification of the enzyme described below.

Methods

Citric acid was determined by the method of Pucher, Sherman, and Vickery (8) as modified by Speck *et al.* (9). *d*-Isocitric and L-malic acids were determined by the polarimetric method as described by Krebs and Eggleston (10).

Although protein concentrations are reported in mg. per cc. in Table I, the concentrations of protein during the course of aconitase fractionation

* National Research Council Fellow, 1946–48. Present address, Department of Physiological Chemistry, University of Pennsylvania Medical School, Philadelphia, Pennsylvania.

† Senior Fellow of the American Cancer Society, 1947–48. Present address, Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts.

were actually followed by optical density measurements at 280 mμ. In order to determine whether or not the protein extinction coefficient at 280 mμ remained constant in succeeding steps, a comparison was made of the original extract and the final preparation. Since the determination of the extinction coefficient at 280 mμ is subject to error due to turbidity, a sample of the diluted extract was centrifuged at about 25,000 R.P.M. in order to clarify the solution. The optical density of this clear supernatant was used to calculate the average extinction coefficient of the soluble proteins. The extinction coefficients ($\epsilon_{280\ m\mu}$ per mg. of protein in a cell with a 1 cm. light path) of the original extract and of the final product were identical

TABLE I

Yields and Degrees of Purification of Aconitase Obtained during Alcohol and Salt Fractionation of Pig Heart Extracts

See the text for the definition of units.

Fraction	Amount of fraction	Protein units		Aconitase units		Total units	Yield, per cent of extract
	cc.	ϵ_{280}	mg. per cc.	units per cc.	units per mg.		
Extract . .	2360	21.2	13.2	132	10.0	310,000	100
1st alcohol fractionation . .	600	18.7	11.7	221	18.9	133,000	42*
2nd " " .	110	23.8	14.9	1010	68	102,000	36
3rd " "	50	15.3	9.6	1260	132	63,000	20
Ammonium sulfate ppt., 0-66%. .	11.4	16.2	10.1	1020	100	11,600	3.7
" " " 66-73%	10.0	25.2	15.7	3700	235	37,000	11.9

* In order to bring the enzyme into precipitated form as rapidly as possible, no effort was made to wash the Filter-Cel pad free of retained protein solution. If this mechanical loss were taken into account, the recovery of enzyme at the end of the first alcohol fractionation would have been 60 per cent.

and equaled 1.6. Protein concentrations were calculated by multiplying the Kjeldahl nitrogen values by 6.25.

Ammonium sulfate concentrations were followed with a modification of the gradient tube method of Linderstrøm-Lang (11). Two bromobenzene-kerosene mixtures having densities of 1.26 and 1.05 were prepared. A 500 cc. glass-stoppered graduate was filled to the 250 cc. mark with the heavier of these two mixtures and the lighter mixture was carefully layered above to the 500 cc. mark. A more or less linear gradient was then produced in the middle third of the tube by vertical stirring in the region above and below the interface. Standard solutions of ammonium sulfate of varying degrees of saturation were introduced as small drops (about 3 c.mm.) along the left side of the gradient. The ammonium sulfate concentration of similar drops of unknown salt content, introduced on the right side of the

tube, could then be read off directly by interpolation. The general theory and manipulation of gradient tubes is described elsewhere (12, 13).

Reagents—Sodium *cis*-aconitate was formed by hydrolyzing the anhydride with 5 per cent sodium carbonate. *cis*-Aconitic anhydride was prepared according to the method of Malachowski and Maslowski (14) and recrystallized from ether-benzene. The final white product melted at 78° (uncorrected). Reagent fumaric acid was used for the fumarase assays. The authors wish to thank Dr. Birgit Vennesland for a gift of triphosphopyridine nucleotide (TPN), which had a purity of 10 per cent.

Definition of Units in Enzyme Determinations

Aconitase (Citrate Formation)—A unit of enzyme activity is defined as the amount of enzyme which forms, at pH 7.4, 1 μM of citric acid from *cis*-aconitic acid (0.025 M) in 15 minutes at 38°.

Fumarase—A unit of enzyme activity is defined as the amount of enzyme at pH 7.4 which forms 1 μM of L-malic acid in 15 minutes at 38°. The polarimetric determination made according to Krebs and Eggleston (10) is carried out in the presence of excess citric and molybdic acids. The specific rotation of the malic-citric-molybdic acid complex is 1340.

Isocitric Dehydrogenase—This determination depends on the reduction of TPN by isocitric acid and the resulting increase in absorption at 340 mμ. 1 unit is defined, according to Mehler *et al.* (15), as the amount of enzyme causing a change in log I_0/I of 0.01 per minute at 340 mμ, calculated for the third 15 second period after the start of the reaction. Conditions for the test system were the same as those of Mehler *et al.* except that 0.3 cc. of 0.1 M tris(hydroxymethyl)aminomethane was used as a buffer instead of glycine. In the present assays, aconitic acid was used as substrate. This was converted to isocitric acid by the action of aconitase in the enzyme preparation. Therefore, the isocitric dehydrogenase activity is a minimum value, since the formation of isocitric acid may be the limiting reaction in the test system. However, this possibility is unlikely in view of the relatively high concentration of aconitase in the final preparation tested.

Properties of Enzyme—In confirmation of the earlier reports of Krebs and Eggleston (3) and of Ochoa (7), aconitase was found to be relatively unstable in aqueous solution. Fractionation of pig heart extracts with ammonium sulfate, although leading to purification of the enzyme, often resulted in considerable losses in the total amount of the enzyme in the various fractions. For this reason, experiments were undertaken to find some stabilizing agent for the enzyme. As previously reported by Krebs and Eggleston (3), glycerol, when added to extracts, retards the rate of decay of the enzyme activity. Cysteine, in the initial stages of the purifica-

tion, likewise showed a stabilized effect. As purification of the enzyme continued, however, glycerol was without effect and cysteine strongly inhibited the enzyme. Of the stabilizing agents tried, citrate or cis-aconitate, the enzyme product or substrate, seemed most effective in protecting the enzyme against decay. The stability of the enzyme in extracts showed great variability from experiment to experiment. Upon including citrate in the homogenizing medium, the enzyme was sufficiently stabilized to permit centrifugation and other manipulatory procedures prior to the first step of purification.

After several steps of purification, it became necessary to dialyze the enzyme prior to electrophoretic studies. In these instances the presence of citrate in the dialyzing medium slowed the rate of decay of the enzyme sufficiently to permit the subsequent electrophoretic separation of the proteins present and the localization of aconitase activity in the cell compartments.

In no instance, however, could stabilizing agents or conditions be found which would protect aconitase against decay to such an extent that dialysis could be used effectively as a step in the preparation of the enzyme.

In a search for reagents which permitted the fractionation of aconitase without large losses of activity through decay, it was found that treatment with aqueous alcohol solutions at low temperatures and ionic strengths resulted in considerable purification. In all cases, absolute alcohol was diluted to 90 per cent before use in fractionations to minimize the heating effect due to dilution. Additions were made slowly and with stirring, special attention being taken to assure that the temperatures of the alcohol-water mixtures were only slightly above their freezing points.

Preparation of Enzyme

Step 1 (Alcohol Fractionation 1)—1 kilo of pig heart,[1] freed of fat and connective tissue, is diced and homogenized in portions in a Waring blendor with 3 liters of buffer (0.002 M sodium citrate, 0.002 M citric acid). The supernatant from the centrifuged homogenate is cooled rapidly in a dry ice-alcohol bath at 0° and alcohol is added to bring the concentration to 15 per cent by volume. Care must be taken to keep the temperature as near as possible to the freezing point of the aqueous alcohol solution. Filter aid[2] is added, the solution is filtered with suction, and the precipitate discarded. In order to assure precipitation of the enzyme by the further addition of alcohol, 1.25 cc. of saturated NaCl are added to each 100 cc. of

---

[1] Although the pig hearts may be used immediately after removal from the animal, they may also be frozen and stored at −15° for as long as 2 weeks without significant loss of enzyme.

[2] Hyflo Super-Cel, Johns-Manville.

15 per cent alcohol filtrate. The alcohol concentration is then slowly increased to 45 per cent, the temperature of the solution being gradually lowered to about −10°. The precipitate is allowed to settle overnight, and the supernatant is siphoned off. The precipitate is centrifuged compactly in a refrigerated centrifuge. The bulk of the enzyme activity is in the precipitate.

Step 2 (Alcohol Fractionation 2)—The precipitate is taken up in sufficient ice-cold water to give an extinction of 20 at a wave-length of 280 mμ in a 1 cm. cell. This corresponds to a protein concentration of about 12.5 mg. of protein per cc. The alcohol in the precipitate is disregarded, and sufficient 90 per cent alcohol (8.5 cc. per 100 cc. of enzyme solution) is added at a gradually diminishing temperature to bring the concentration to 8 volumes per cent. The inactive precipitate is centrifuged off in a refrigerated centrifuge. Alcohol is then added to the supernatant to bring the alcohol concentration to 23 per cent. The temperature is meanwhile lowered to −7° to −8°. The solution is centrifuged and the supernatant discarded.

Step 3 (Alcohol Fractionation 3)—The precipitate is taken up in sufficient ice-cold water to give an extinction of 24 at 280 mμ in a 1 cm. cell (15 mg. of protein per cc.). The alcohol content of the precipitate is disregarded, sufficient alcohol is added to bring the concentration to 8 per cent, and the precipitate is centrifuged off and discarded. The pH of the supernatant, which is about 5.6, is then adjusted to 6.8 by the careful addition of 5 per cent sodium carbonate. Alcohol is added to 23 per cent and the precipitate, containing the activity, centrifuged off.

Step 4 (Ammonium Sulfate Fractionation)—The precipitate from Step 3 is taken up in 0.1 M phosphate buffer, pH 7.4, containing 0.05 M sodium citrate (extinction of protein solution at 280 mμ = 16, equivalent to 10 mg. of protein per cc.). Ammonium sulfate is added slowly until the solution is 66 per cent saturated.[3] During the addition, the temperature is lowered progressively to −10°. The precipitate is centrifuged[4] for 5 minutes at 8000 R.P.M. and the precipitate discarded. This centrifugation is carried out at −5° to −10°. Ammonium sulfate is then added to the supernatant until the solution is 73 per cent saturated.[3] The precipitate is centrifuged as above and the supernatant discarded. The precipitate contains the bulk of the enzyme, which has been purified about 23-fold. In the precipitated form, it may be frozen with dry ice and stored for a relatively long time without loss in activity.

If further purification of the enzyme is attempted, it is probably inadvisable to use ammonium sulfate in any other than the last step, since salts

[3] Referred to saturated ammonium sulfate at 20°.
[4] Sorvall angle centrifuge, Ivan Sorvall, Inc., New York.

cannot be removed by dialysis without loss of enzyme activity. Moreover, such high concentrations of alcohol are required to precipitate the enzyme in the presence of salts that denaturation results. In Table I are summarized the yields and degrees of purification obtained at each step in the preparation of the enzyme.

Tests of Purity

The purity of the final preparation was studied by electrophoretic analysis and by enzymatic and spectrophotometric tests for fumarase, isocitric dehydrogenase, and hemoglobin + myoglobin.

Electrophoresis—A preparation of enzyme with an activity-protein ratio of 235 was dialyzed against a buffer at pH 7.4, containing 0.033 M phosphate and 0.0166 M sodium citrate and having a specific conductance of 0.00499. Electrophoresis (220 volts, 19 ma.) was permitted to proceed for 5 hours, during which time the proteins had separated into three non-homogeneous components. By enzymatic analysis of the chambers of the electrophoresis cell, it was determined that the aconitase activity was associated with the slowest of these three boundaries, having a mobility of 1.18×10^{-5} (ascending boundary). A red pigment, either hemoglobin or myoglobin, or both, was also associated with this electrophoretic component.

If one assumes that either hemoglobin or myoglobin or both constitute the only impurities in this active component, it may be calculated from the electrophoretic diagram and from the hemoprotein estimations (see below) that the purity of aconitase in the final ammonium sulfate fraction is about 30 per cent. This figure represents the maximum value, since the presence of impurities with similar mobilities cannot be excluded on the basis of the present data.

Spectrophotometric Assay of Hemoproteins—Total hemoglobin plus myoglobin was estimated in the electrophoretic compartment containing the aconitase activity. After treatment with carbon monoxide, readings were made at the isosbestic point of these two proteins (575.5 mμ) according to de Duve (16). The hemoprotein content was 21.9 per cent of the total protein in the active electrophoretic component.

Enzymatic Tests—The enzyme activities of two enzymes of the tricarboxylic acid cycle, isocitric dehydrogenase and fumarase, were followed during the purification procedure.

Isocitric dehydrogenase was found in rather high concentrations in the final preparation of aconitase which had undergone four steps of purification. Assay of the original extract was subject to so many errors that a value for the enzyme activity was not obtained on this sample. The final enzyme preparation, however, contained 480 units of enzyme per mg. of protein. Ochoa (7) has found that acetone powders of pig heart contain

isocitric dehydrogenase in concentration of 167 units per mg. of protein and was able to purify this preparation about 4-fold (670 units per mg. of protein). Isocitric dehydrogenase is thus, probably, a significant impurity in the aconitase preparation.

The concentration of fumarase at various steps in the fractionation of aconitase was determined. While aconitase was purified 23-fold, the concentration of fumarase had fallen from 110 units per mg. of protein in the original extract to 18.3 units per mg. in the final preparation. It is thus apparent that fumarase, although present in the final preparation, is not a very large impurity.

The possibility that "aconitase" may be two enzymes, one catalyzing citric and one isocitric formation, has been a subject of discussion (4, 5). Evidence gained from these experiments does not support this conclusion. To the contrary, the ratio of the activity of the enzyme in forming citric and isocitric acids (as measured by the polarimetric method of Krebs and Eggleston (10)) from cis-aconitic acid has remained constant, within the limits of experimental error, during the entire fractionation procedure. In addition, the activities of the enzyme preparation in respect to both citric and isocitric acid formation, appear to decay at the same rate and are not separable under the electrophoretic conditions employed here. However, definite proof that the two reactions are catalyzed by the same enzyme must await complete purification.

SUMMARY

Aconitase has been purified 23-fold by low temperature alcohol and ammonium sulfate fractionation in the presence of citric acid as a stabilizing agent. Electrophoretic examination of this preparation and subsequent enzyme analysis of the electrophoresis cell chambers indicate a maximum purity of about 30 per cent. The preparation contains hemoprotein and also exhibits considerable isocitric dehydrogenase activity. Fumarase is present, but only as a minor impurity. The data do not suggest the presence of two aconitases.

BIBLIOGRAPHY

1. Martius, C., and Knoop, F., Z. physiol. Chem., 246, p. I (1937).
2. Breusch, F. L., Z. physiol. Chem., 250, 262 (1937).
3. Krebs, H. A., and Eggleston, L. V., Biochem. J., 38, 426 (1944).

4. Jacobsohn, K. P., and Soares, M., *Compt. rend. Soc. biol.*, **131**, 642 (1939).
5. Jacobsohn, K. P., Soares, M., and Tapadinkas, J., *Bull. Soc. chim. biol.*, **22**, 48 (1940).
6. Johnson, W. A., *Biochem. J.*, **33**, 1046 (1939).
7. Ochoa, S., *J. Biol. Chem.*, **174**, 133 (1948).
8. Pucher, G. W., Sherman, C C., and Vickery, II. B., *J. Biol. Chem.*, **113**, 235 (1936).
9. Speck, J. F., Moulder, J. W., and Evans, E. A., Jr., *J. Biol. Chem.*, **164**, 119 (1946).
10. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **37**, 334 (1943).
11. Linderstrøm-Lang, K., Jacobsen, O., and Johansen, G., *Compt.-rend. trav. Lab. Carlsberg*, **23**, 17 (1938).
12. Lowry, O. H., and Hastings, A. B., *J. Biol. Chem.*, **143**, 257 (1942).
13. Anfinsen, C. B., in Wilson, D. W., Preparation and measurement of isotopic tracers, Ann Arbor (1946).
14. Malachowski, R., and Maslowski, M., *Ber. chem. Ges.*, **61**, 2521 (1928).
15. Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S., *J. Biol. Chem.*, **174**, 961 (1948).
16. de Duve, C., *Acta chem. Scand.*, **2**, 264 (1948).

THE CONDENSATION OF C¹⁴-NITROMETHANE WITH
D-ARABINOSE: PREPARATION OF 1-C¹⁴-D-GLUCOSE
AND 1-C¹⁴-D-MANNOSE

By JOHN C. SOWDEN

*(From the Radiochemistry Laboratory, Department of Chemistry,
Washington University, St. Louis)*

The incorporation of isotopic C^{11}, C^{13}, or C^{14} into the D-glucose molecule has been accomplished previously only through the agency of growing plants (1–3) or of living animals (4–6). The D-glucose obtained from plants grown in an atmosphere containing carbon¹⁴ dioxide was found to be labeled in all positions of the carbon chain, with the highest concentration of the radioactivity in carbons 3 and 4 of the hexose (7). The glycogen produced by rats, when injected with C^{13}-carbonate, has been hydrolyzed to isotopic D-glucose labeled only in carbons 3 and 4 (5).

It was obviously desirable to make available, in addition to the biosynthetic labeled sugar, D-glucose and other sugars containing isotopic carbon only in one known position of the carbon chain, preferably in the terminal aldehyde group. Sugars labeled in this manner should be valuable in the interpretation of experiments that involve fragmentation of the molecule, as in the various fermentations or the assimilation of the carbohydrates.

By application of the nitromethane synthesis (8) to D-arabinose, radioactive D-glucose and D-mannose containing C^{14} in only the aldehyde carbon have now been prepared in the crystalline state (9). By condensing C^{14} nitromethane, with radioactivity of approximately 8 mc. per mole, and D-arabinose under alkaline conditions, 1-nitro-1-desoxysorbitol and 1-nitro-1-desoxy-D-mannitol were obtained in the crystalline state in an over-all yield of approximately 28 per cent, based on nitromethane. After separation of the epimeric nitro alcohols by fractional crystallization, their sodium salts were treated with sulfuric acid to produce the corresponding hexoses, D-glucose and D-mannose in good yield. The hexoses showed radioactivity of approximately 62,000 counts per minute per mg. While this activity will allow considerable dilution in subsequent experiments before accurate measurement becomes impossible, the synthesis should be applicable to the preparation of aldose sugars with even higher specific activities.

EXPERIMENTAL

C^{14}-*Nitromethane*—16 gm. of C^{14}-methanol containing 4 mc. of radioactivity[1] were converted to methyl iodide by distillation with hydriodic

---

[1] Oak Ridge National Laboratory, Oak Ridge, Tennessee.

acid (10).   The yield of product after drying over calcium chloride and re-distillation was 59 gm. (83 per cent).

The methyl iodide was added dropwise to an intimate mixture of 120 gm. of silver nitrite (Mallinckrodt) and 250 gm. of dry sand under a cold finger reflux condenser maintained at −40° by means of dry ice and chloroform.   When the addition of methyl iodide was complete, the mixture was heated on the steam bath for 2 hours.   The reflux condenser was then warmed to −5° and the resulting gaseous methyl nitrite was flushed from the system past the reflux condenser, with a slow current of dry air, into a receiver at −70°.   The yield of methyl nitrite was 4 gm. (16 per cent).

The residual nitromethane was distilled from the reaction flask in a slow current of dry air into a receiver containing 20 gm. of silver nitrite and 50 gm. of calcium sulfate (drierite).   Redistillation then yielded 17.9 gm. (71 per cent) of nitromethane (11).

1-C^{14}-1-Nitro-1-desoxysorbitol and 1-C^{14}-1-Nitro-1-desoxy-D-mannitol—The above 17.9 gm. of nitromethane were added to a suspension of 44 gm. of D-arabinose in 50 cc. of absolute methanol and the mixture was treated with a solution of sodium methoxide containing 8.8 gm. of sodium in 350 cc. of absolute methanol.   After vigorous stirring for 5 hours at room temperature in a closed system, the precipitated sodium nitro alcohols were filtered and washed in rapid succession with cold methanol (−20°), ether, and petroleum ether.   The moist powder was then dissolved in 350 cc. of ice water and passed immediately through a column containing approximately 1 kilo of Amberlite IR-100-AG ion exchange resin in the hydrogen form.[2]   Concentration of the effluent (1300 cc.) at reduced pressure, followed by addition of a few cc. of ethanol to the resulting sirup, yielded the crystalline nitro alcohols admixed with unchanged D-arabinose.   After filtration and washing with a small amount of cold ethanol, the yield of crude, air-dry crystals was 30 gm.[3]

The nitro alcohols were extracted from the crude product in a Soxhlet apparatus with dry acetone for several hours, leaving a residue of 10.5 gm. of D-arabinose, m.p. 156–159°.   Concentration of the acetone extract and fractional crystallization of the residue from ethanol then yielded 1.1 gm. of D-arabinose, m.p. 150–160°, 11.0 gm. of 1-nitro-1-desoxy-D-mannitol, m.p. 130–133°, and 6.1 gm. of 1-nitro-1-desoxysorbitol, m.p. 103–105°.[4]

[2] The Resinous Products and Chemical Company, Philadelphia, Pennsylvania.

[3] Occasionally, subsequent fractionation of the crystalline product may show that only 1-nitro-1-desoxy-D-mannitol and unchanged D-arabinose have crystallized at this stage.   In this event, relatively pure 1-nitro-1-desoxysorbitol may be recovered from the mother liquor by concentration followed by the addition of ether.

[4] The conditions for the fractional crystallization are largely empirical.   The main bulk of the 1-nitro-1-desoxy-D-mannitol crystallizes first admixed with a negligible amount of D-arabinose.   Succeeding crops then usually alternate between the more

The recorded (8) constants for the pure enantiomorphic nitro alcohols are 1-nitro-1-desoxy-L-mannitol, m.p. 133–134°, and 1-nitro-1-desoxy-L-glucitol, m.p. 107–108°.

1-C^{14}-D-Mannose—5 gm. of the above 1-nitro-1-desoxy-D-mannitol were dissolved in 15 cc. of 2 N sodium hydroxide and added dropwise to a stirred solution of 7.5 cc. of sulfuric acid in 9 cc. of water at 10–15°. The resulting solution was diluted to 100 cc. with water and just neutralized with solid sodium carbonate. Phenylhydrazine (3 cc.) in 7 cc. of acetic acid was then added. After standing overnight at 0°, the precipitated hydrazone was filtered and washed with water, ethanol, and ether. The yield was 5.0 gm. (78 per cent), m.p. 190–191°.

Cleavage of the hydrazone with benzaldehyde in the usual manner yielded 2.45 gm. of crystalline D-mannose, m.p. 127–131°. (0.9 gm. of mannose phenylhydrazone was recovered from the mother liquors; yield, 90 per cent.)

1-C^{14}-D-Glucose—3 gm. of the above 1-nitro-1-desoxysorbitol in 9 cc. of 2 N sodium hydroxide were added dropwise to a stirred solution of 4.5 cc. of sulfuric acid in 5.5 cc. of water at 10–15°. After dilution to 125 cc. with water, the solution was just neutralized to Congo red with barium hydroxide solution and filtered. The solution was then passed successively through two columns containing respectively 100 gm. of Amberlite IR-100-AG[2] and 100 gm. of Duolite A-4.[3] The sirup obtained on concentration crystallized readily when mixed with a few drops of ethanol and warmed at 50°. The yield of D-glucose was 1.5 gm. (58 per cent), m.p. 142–145°. The residual sirup from the first crystallization was redissolved with 2 gm. of non-radioactive D-glucose and yielded 2.0 gm. of radioactive glucose, m.p. 145–146°, showing 14 per cent of the activity of the more active sample above.

Measurement of Radioactivity—The sugars were mounted directly as thin films (about 20 γ per sq. cm.) on stainless steel disks by evaporation with ethanol and counted in the RCL nucleometer (Radiation Counter Laboratories, Chicago). The observed activity was 62,000 ± 2000 counts per minute per mg.

SUMMARY

C^{14}-Methyl iodide has been converted to C^{14}-nitromethane and C^{14}-methyl nitrite by the Victor Meyer reaction with silver nitrite.

C^{14}-Nitromethane has been condensed with D-arabinose to give radioactive 1-nitro-1-desoxysorbitol and 1-nitro-1-desoxy-D-mannitol.

soluble 1-nitro-1-desoxysorbitol and 1-nitro-1-desoxy-D-mannitol. Seeding crystals of the pure isomers may be used advantageously.

[3] Chemical Process Company, San Francisco, California. Before use, this material was treated with several volumes of 5 per cent sodium chloride solution and then washed with distilled water until chloride-free.

The sugar nitro alcohols have been converted to crystalline D-mannose and D-glucose containing C^{11} in the aldehyde carbon.

BIBLIOGRAPHY

1. Livingston, L G., and Medes, G., *J. Gen. Physiol.*, **31**, 75 (1947).
2. Aronoff, S., Benson, A., Hassid, W. Z., and Calvin, M., *Science*, **105**, 644 (1947).
3. Putman, E. W., Hassid, W. Z , Krotkov, G , and Barker, H. A., *J. Biol. Chem.*, **173**, 785 (1948)
4. Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140**, 171 (1941).
5. Wood, H. G., Lifson. N., and Lorber, V., *J. Biol. Chem.*, **159**, 475 (1945).
6. Zilversmit, D. B , Chaikoff, I L., Feller, D. D., and Masoro, E. J., *J. Biol. Chem.*, **176**, 389 (1948).
7. Aronoff, S., Barker, H. A., and Calvin, M., *J. Biol. Chem.*, **169**, 459 (1947).
8. Sowden, J. C., and Fischer, H. O. L., *J. Am. Chem. Soc.*, **66**, 1312 (1944); **67**, 1713 (1945); **68**, 1511 (1946); **69**, 1048, 1963 (1947).
9. Sowden, J. C., *Science*, **109**, 229 (1949).
10. Norris, J. F., *Am Chem. J.*, **38**, 639 (1907).
11. Meyer, V., *Ann. Chem* , **171**, 23 (1874).

STUDIES OF THE MECHANISM OF HISTIDINE SYNTHESIS IN LACTIC ACID BACTERIA*

BY HARRY P. BROQUIST AND ESMOND E. SNELL

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

Little is known of the mechanism of biosynthesis of histidine. The occurrence of the imidazole ring in both histidine and the purine bases suggests a relationship between these metabolites. It was early found that ingestion of histidine increased the excretion of allantoin in rats, and on this basis it was postulated that histidine might serve as a precursor of the purine bases (1, 2). More recent experiments, however, seem to exclude this possibility (3–5). The converse relationship, *i.e.* that the purine bases might serve as precursors of histidine, was envisaged (6), but no evidence in support of this idea was found, since rats failed to show increased growth when purine bases were added to a histidine-free ration (6).

Appropriate studies with *Lactobacillus arabinosus* showed that under appropriate conditions histidine and purine bases showed a common effect in promoting growth. Further studies of this phenomenon with several bacteria fail to support the view that histidine functions as a precursor for synthesis of purine bases, but indicate strongly that the purine bases do serve as precursors in the synthesis of histidine. Vitamin B_6 is required for synthesis of histidine in these organisms; data given below demonstrate that it is necessary for conversion of imidazolepyruvic acid to histidine.

Methods

Cultures and Media—*Lactobacillus arabinosus* 17-5, *Lactobacillus casei*, and *Streptococcus faecalis* R were used in this study. Methods for care of stock cultures and preparation of inocula have been described (7). A synthetic medium which permitted the elimination of histidine, purine bases, or vitamin B_6 was employed. The basal medium used for *L. arabi-*

nosus and *L. casei* is given in Table I. For culture of *S. faecalis*, the sodium acetate of this medium was omitted and the concentration of sodium citrate, K_2HPO_4, and KH_2PO_4 were increased to the levels employed by Rabinowitz and Snell (9). Time and temperatures of incubation are given in Tables II to IV; growth was measured turbidimetrically with the Evelyn colorimeter.

TABLE I

Composition of Basal Medium for L. arabinosus and L. casei *

Component	Amount per 100 ml. of double strength medium	Component	Amount per 100 ml. of double strength medium
	gm.		γ
Glucose	2.0	Riboflavin	40
Sodium citrate (U. S. P. XII)	2.0	Pyridoxal hydrochloride	20
" acetate (anhydrous)	1.2	Calcium pantothenate	40
KH_2PO_4	0.6	Thiamine chloride	20
K_2HPO_4	0.6	Nicotinic acid	40
	mg.	Biotin	0 2
$MgSO_4 \cdot 7H_2O$	100	Pteroylglutamic acid	2 0
$MnSO_4 \cdot H_2O$	100		*mg.*
NaCl	2	DL-Alanine	200
$FeSO_4 \cdot 7H_2O$	2	DL-Aspartic acid	200
Adenine sulfate	2	L-Glutamic acid	200
Guanine hydrochloride	2	L-Arginine hydrochloride	40
		L-Lysine hydrochloride	40
		Other amino acids†	
		L forms or	20 each
		DL forms	40 "

* The composition of the medium is based on that of Henderson and Snell (8) and that of Williams, Broquist, and Snell (7).

† Cystine, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine.

Preparation of β-Imidazolepyruvic Acid—2-Phenyl-4-(1-acetylimidazolyl-4(or 5)-methylidene)-oxazolone was prepared as described by Pyman (10) by condensation of hippuric acid with 4(5)-imidazolealdehyde in the presence of sodium acetate and acetic anhydride. This compound (1 gm.) was hydrolyzed by refluxing for 2.5 hours with 40 ml. of 6 N HCl. After cooling, the benzoic acid was removed by ether extraction. The aqueous residue was concentrated to dryness *in vacuo*, and the residue extracted with 5 ml. of hot ethanol. The ethanol extract was concentrated *in vacuo* to dryness, dissolved in 3 ml. of water, cooled in an ice bath, and NaOH

added dropwise, with stirring. Imidazolepyruvic acid precipitates near its isoelectric point, and was filtered, washed with acetone, and dried. Yield, 150 mg. (30 per cent of theory); neutralization equivalent found, 156; calculated, 154. Additional keto acid can be obtained from the mother liquors.

As reported by others (11) this compound does not show a good melting point; for further characterization it was converted to the 2,4-dinitrophenyl-hydrazone. The first product, obtained in quantitative yield, is a hydrated 2,4-dinitrophenylhydrazone of imidazolepyruvic acid hydrochloride (12). After recrystallization it decomposed at 190–192°. On heating for 50 hours at 140° and 1 mm. pressure over calcium chloride this product was converted to the 2,4-dinitrophenylhydrazone of imidazolepyruvic acid

TABLE II

Relationships between Purine Bases, Histidine, and Vitamin B_6 in Growth of L. arabinosus

Omissions from basal medium	Pyridoxal present		Pyridoxal omitted	
	Per cent of incident light transmitted*			
	36 hrs.	60 hrs.	36 hrs.	60 hrs.
None	24	24	25	25
Adenine and guanine.....	24	25	39	39
Histidine	25	25	98	93
Adenine, guanine, and histidine	99	38	97	96

* Uninoculated medium = 100; incubated at 37°.

which decomposed at 240°. Stumpf and Green (12) report 192° and 239° respectively, as the decomposition points of these two compounds.

Results

Data summarizing the interrelationships of histidine, purine bases, and vitamine B_6 in growth of *L. arabinosus* are summarized in Table II. In the presence of vitamin B_6 (pyridoxal) neither an external source of histidine nor of purine bases is essential for rapid growth; when both are omitted together, however, growth is markedly retarded. During the first 36 hours of incubation, therefore, addition of *either* histidine *or* purine bases has the same marked effect upon growth. These results suggest a product-precursor relationship between histidine and the purine bases as illustrated by the equation

$$\text{Purine base} \qquad\qquad \text{histidine} \qquad\qquad (1)$$

but give no evidence of whether the reaction proceeds according to Reaction 1, *a* or *b*. When either the product or a readily utilizable precursor of it is supplied, rapid growth occurs; when both are absent and must be synthesized, growth is much slower.

In the absence of added vitamin B_6, histidine becomes essential for growth of this organism; *i.e.*, it can no longer be synthesized (Table II). Under these conditions, the purine bases do not replace histidine. Thus, in accordance with the previous report of Lyman *et al.* (13), the vitamin
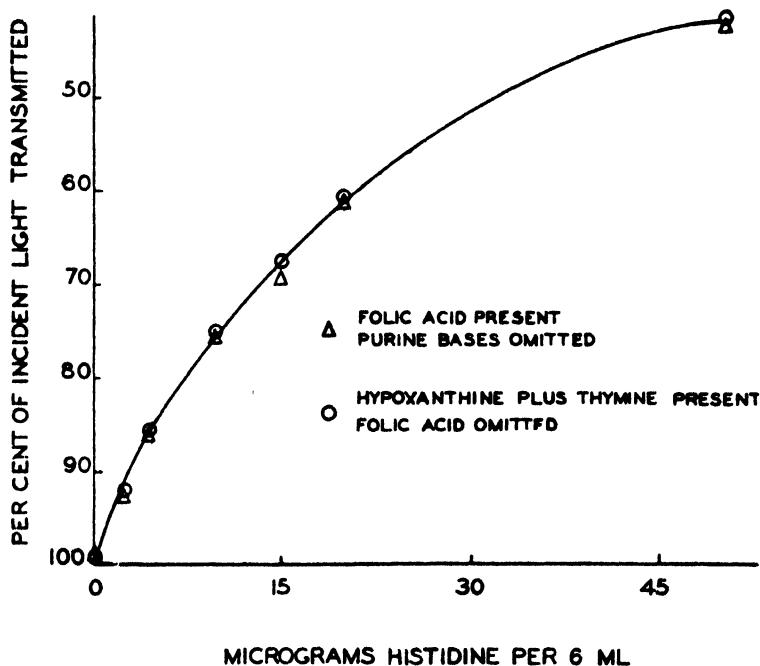
FIG. 1. Comparative histidine requirement of *S. faecalis* in the presence and absence of purine bases. △ 60 mμgm. of pteroylglutamic acid per 6 ml.; ○, 200 γ of hypoxanthine and 60 γ of thymine per 6 ml. Incubated 24 hours at 37°.

B_6 requirement of *L. arabinosus* is greatly enhanced by the absence of histidine from the medium, apparently because vitamin B_6 is required in its synthesis.

The possibility that histidine might serve as a precursor for purine bases (Reaction 1, *b*, above) was first considered. The nutritional requirements of *S. faecalis* permitted an examination of this possibility. This organism requires either folic acid or a mixture of thymine and purine bases for growth (14–16). In the presence of folic acid, therefore, it can

synthesize purine bases; in the absence of folic acid it cannot, and must have them preformed in the medium. If histidine, which is required preformed by this organism, serves as a precursor of purine bases, then the requirement of the organism for histidine should be increased when the organism is cultured under conditions such that it must synthesize purine bases. The histidine requirement of *S. faecalis* was therefore compared in a medium which contained folic acid, but no purine bases, and a medium which contained thymine and hypoxanthine in place of folic acid. The results (Fig. 1) show that the histidine requirement is identical in both cases. Histidine does not, therefore, serve as a precursor for purine synthesis in this organism, a conclusion in full agreement with that reached by modern techniques in higher animals (3–5).

To test the alternative hypothesis, that the purine bases might serve as precursors in the synthesis of histidine (Reaction 1, *a*), *L. casei* was used. This organism does not have an absolute requirement for histidine (17); and after successive subcultures in the presence of decreasing amounts of histidine the organism grew well without added histidine. The folic acid requirement of this organism can also be eliminated by a mixture of purine bases and thymine,[1] which are essential for growth in the absence of folic acid (15). By omitting folic acid from the medium, but including excess thymine, the organism responds to graded levels of purine bases. Thus, as is shown in Fig. 2, it was possible to determine the requirement of *L. casei* for the purine base guanine in the presence and absence of histidine. Significantly higher amounts of guanine were required by the organism under conditions in which histidine had to be synthesized. This was interpreted to mean that some of the guanine was being utilized in the biosynthesis of histidine, in accordance with Reaction 1, *a*, above.

Indirect evidence strengthening the view that purine bases function as precursors in synthesis of histidine was obtained by "inhibition analysis" (18). It was found that imidazolealdehyde was markedly inhibitory for growth of *L. arabinosus* under certain conditions. Thus, as is shown in Curve 1, Fig. 3, if both the purine bases and histidine were omitted from the medium, less than 100 γ of imidazolealdehyde completely inhibited growth of *L. arabinosus*. If histidine was now added at a level sufficient for maximum growth of the organism, imidazolealdehyde was less effective as a growth inhibitor, as shown in Curve 2, Fig. 3. If the histidine level was now increased 50-fold, the degree of inhibition by imidazolealdehyde was not further affected, indicative of a non-competitive relationship be-

[1] Various authors (*e.g.* (17)) have reported only partial replacement of folic acid by purine bases and thymine for this organism. If purine bases and thymine are added in sufficient amounts, however, growth with them is essentially as good as with folic acid.

tween histidine and imidazolealdehyde.   When histidine was omitted from the medium and a mixture of purine bases sufficient for maximum growth of the organism was added, imidazolealdehyde became markedly less effective as a growth inhibitor as shown in Curve 3, Fig. 3.   Lastly, if excess histidine was added in addition to the purine bases of Curve 3, the inhibitor was completely ineffective (Curve 4, Fig. 3).   Although the sites of action of this inhibitor are not fully known, these data are consistent with the view that at low concentrations the inhibitor interferes primarily with
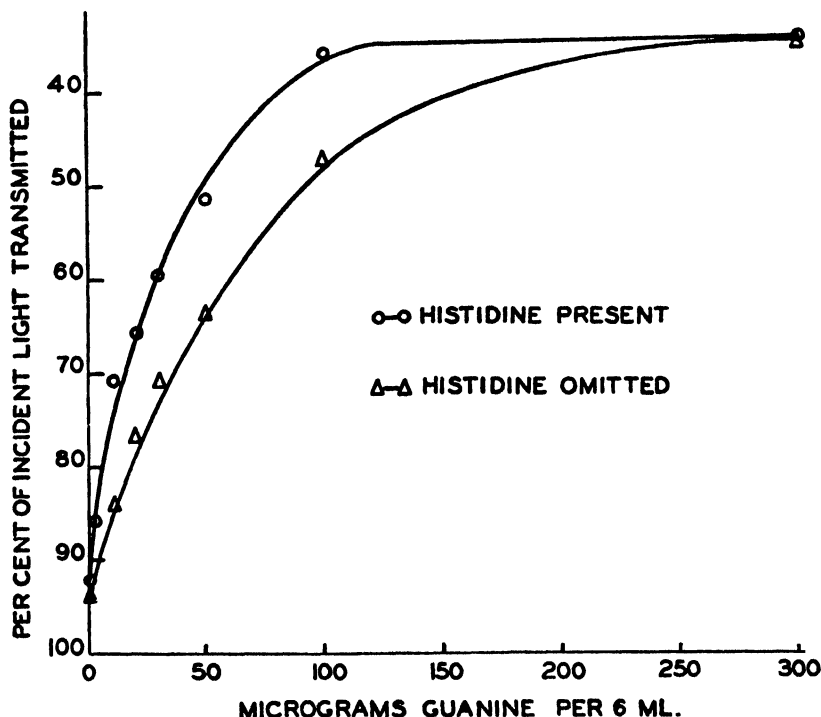
FIG. 2. The sparing action of histidine on the requirement of *L casei* for purine bases.   Incubated 72 hours at 37°.

synthesis of the purine bases, while at higher concentrations it also prevents transformation of purine bases to histidine.   At low concentrations, therefore, its effectiveness as an inhibitor is markedly reduced by addition of purine bases, since when these are present the inhibited reaction is no longer essential for growth.   The limited action of histidine in alleviating toxicity at these low levels of the inhibitor (Curve 2, Fig. 3) results from its sparing action on the amount of purine bases necessary for growth. At higher concentrations of inhibitor, purine bases alone are no longer

fully effective in overcoming the inhibitory action, since at these concentrations the inhibitor also prevents transformation of purine bases to histidine. Addition of the product of this inhibited reaction, histidine, to the medium then renders the inhibitor wholly ineffective.

The comparative activities of various purine bases and related products in promoting growth of L. arabinosus in a medium free of both histidine and purine bases were next determined (Table III). Histidine itself is most
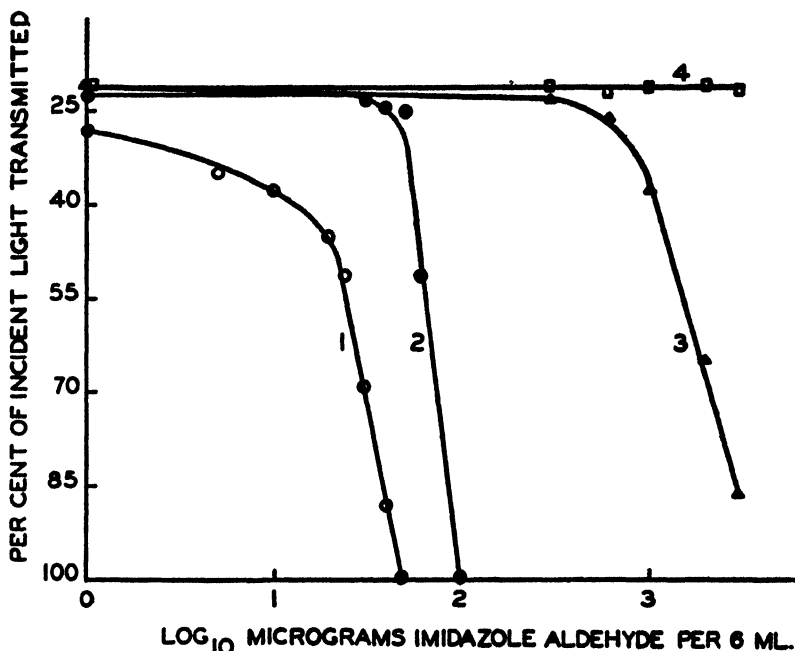
FIG. 3. Effect of purine bases and histidine on inhibition of growth of L. arabinosus by imidazolealdehyde. Curve 1, no histidine or purine bases in medium; Curve 2, 200 γ of histidine, no purine bases; Curve 3, 50 γ each of adenine sulfate, guanine hydrochloride, xanthine, and hypoxanthine, no histidine; Curve 4, as in Curve 3 plus 200 γ of histidine. Incubated 64 hours at 37°.

effective in promoting growth under these conditions, but is approached very closely by xanthine and guanine. Adenine and hypoxanthine are much less effective over the shorter time interval (36 hours), but with sufficient time (48 hours) show full effectiveness. Separate trials showed that adenosine, inosine, and guanosine were never more, and were sometimes less, active in promoting growth under these conditions than the parent purine bases, suggesting their conversion to the purine bases before utilization for histidine synthesis. 5(4)-Amino-4(5)-imidazole carboxamide,

a proposed intermediate in purine synthesis (19), also stimulated growth of *L. arabinosus* in the histidine-deficient medium, but was less effective than any of the purine bases. This suggests that it is first converted to a purine base, after which histidine synthesis occurs. Allantoin was ineffective, while uric acid was somewhat inhibitory.

Under the above growth conditions, which require synthesis of histidine, xanthine is the most active purine base, whereas hypoxanthine is one of the least active. In previous reports of the relative activity of the purine bases in promoting growth of *L. arabinosus* in complete media (in which histidine synthesis was unnecessary), hypoxanthine was more effective in promoting growth than xanthine or other purine bases (20, 21). The growth curves of *L. arabinosus* in Fig. 4 emphasize the differences in biological activity that can be demonstrated for hypoxanthine and xanthine, depending on whether or not the organism must synthesize histidine. In

TABLE III

Comparative Activities of Various Purine Bases and Related Compounds in Promoting Growth of L. arabinosus in Histidine-Free Medium

Amount per 6 ml.	Hypoxanthine		Adenine		Guanine		Xanthine		Histidine	
	Per cent of incident light transmitted*									
	36 hrs.	48 hrs.	36 hrs	48 hrs.	36 hrs.	48 hrs.	36 hrs.	48 hrs.	36 hrs.	48 hrs.
γ										
0	100	89	100	89	100	89	100	89	100	89
3	100	74	98	72	96	82	89	83	90	86
30	100	47	97	17	62	49	56	43	50	48
300	97	28	97	29	25	25	27	26	24	24

* Uninoculated medium = 100; incubated at 37°.

a medium deficient in both purine bases and histidine there was a very marked lag phase in the growth of *L. arabinosus*, as shown by Curve 6. If histidine was present in the medium, growth was considerably accelerated (Curve 3). Furthermore if both purine and histidine were present, then hypoxanthine (Curve 1) was more stimulatory than xanthine (Curve 2). Although the difference in activity of the two purine bases was not large, repeated trials consistently favored hypoxanthine over xanthine in promoting early growth of *L. arabinosus* under these conditions. In marked contrast, however, was the finding that in the absence of histidine, *i.e.* under conditions requiring histidine synthesis, xanthine (Curve 4) was markedly superior to hypoxanthine (Curve 5) in promoting early growth of the organism. These data and those of Table III indicate that xanthine is the preferred precursor for histidine synthesis, although other purine bases will also serve.

The data of Table II show that when vitamin B$_6$ was omitted from the medium, histidine was required by *L. arabinosus*, even though purine bases were in the medium. This was interpreted to mean that vitamin B$_6$ is involved in the synthesis of histidine from purine bases. Several compounds were examined as possible intermediates in this synthesis by testing them for growth-promoting action in a histidine-free medium in the presence and absence of vitamin B$_6$. The basal medium (Table I) from
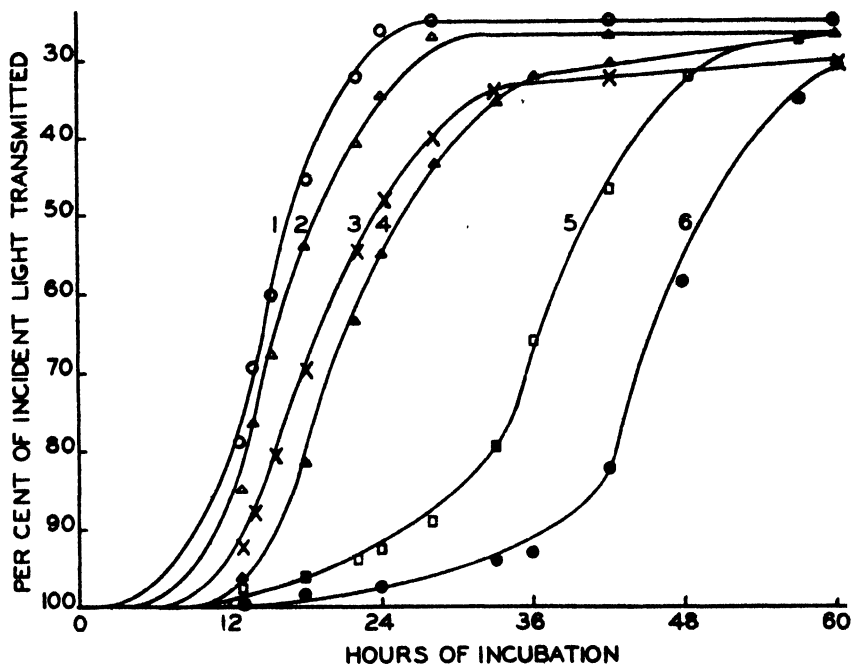
Fig. 4. Comparative activities of xanthine and hypoxanthine in promoting growth of *L. arabinosus* in the presence and absence of histidine. Curve 1, hypoxanthine plus histidine; Curve 2, xanthine plus histidine; Curve 3, histidine with purines omitted; Curve 4, xanthine with histidine omitted; Curve 5, hypoxanthine with histidine omitted; Curve 6, histidine and purine bases omitted. All compounds were added at a level of 500 γ per 6 ml. Incubation at 37°.

which histidine, purine bases, and pyridoxal were omitted sufficed for this purpose with *L. arabinosus*. With *S. faecalis*, which requires histidine even in the presence of the purine bases, only histidine and pyridoxal were omitted. *S. faecalis* requires D-alanine for growth in the absence of vitamin B$_6$ (22); an excess of this compound was therefore added to both vitamin B$_6$-deficient media. The results are shown in Table IV. Histidine promoted growth of both organisms even though vitamin B$_6$ was

omitted from the medium. Imidazole, imidazolecarboxylic acid, imidazoleacrylic acid, imidazolelactic acid, and histamine were all inactive under the conditions of assay. These compounds are probably not intermediates, therefore, in the conversion of purine bases to histidine. Imidazolepyruvic acid, however, was utilized for histidine synthesis by both organisms when vitamin B_6 was present, but not when this vitamin was absent. This keto acid may well be a normal intermediate, therefore, in the synthesis of histidine. The fact that purine bases duplicate the

TABLE IV

Growth-Promotion by Substituted Imidazoles in Presence and Absence of Vitamin B_6

Additions to 6 ml. of histidine-free medium*	S. faecalis		L. arabinosus	
	Pyridoxal present	Pyridoxal omitted	Pyridoxal present	Pyridoxal omitted
	Per cent of incident light transmitted†			
None..	100	100	76	97
Histidine (1 mg.).	55	57	25	42
Imidazole (1 ")	100	100	76	97
Imidazolecarboxylic acid (1 mg.).......................	100	100	75	
Imidazoleacrylic acid (1 mg.)..	100	100	75	97
Imidazolelactic " (1 ")	100	100	75	94
Imidazolepyruvic " (1 ")	51	99	35	97
Histamine (1 mg.)........	100	100	76	97
Reaction product of imidazolepyruvic acid and glutamic acid‡....	52	62		

* Purine bases were also omitted from the medium for L. arabinosus, but not for S. faecalis (see the text).

† Uninoculated medium = 100. S. faecalis was incubated for 24 hours at 37°; L. arabinosus for 36 hours at 37°.

‡ 3 mg. of imidazolepyruvic acid and 90 mg. of glutamic acid in 6 ml. of water at pH 7.0 were boiled under a reflux for 8 hours. Aliquots equivalent to 0.5 mg. of initial imidazolepyruvic acid were assayed.

growth-promoting action of histidine for L. arabinosus only when vitamin B_6 is present (Table II) may result wholly or in part from the essential rôle of this vitamin in converting imidazolepyruvic acid into histidine. Although the mechanism of the latter conversion is unknown, the known function of vitamin B_6 in catalyzing transamination reactions (23, 24) makes this a logical route. That imidazolepyruvic acid can be transformed into histidine by chemical transamination is shown by the last line of Table IV. The reaction product obtained when imidazolepyruvic acid

was refluxed with excess glutamic acid promoted growth of *S. faecalis* in the absence of vitamin B_6, indicating that histidine had been formed.

A quantitative comparison of the response of *S. faecalis* to histidine and imidazolepyruvic acid in the presence and absence of vitamin B_6 is shown in Fig. 5. With vitamin B_6 in the medium, imidazolepyruvic acid was only slightly less active than was L-histidine. The marked difference in response of *S. faecalis* to histidine and imidazolepyruvic acid in the absence of vitamin B_6 (Curves 2 and 4, Fig. 5, respectively) provides
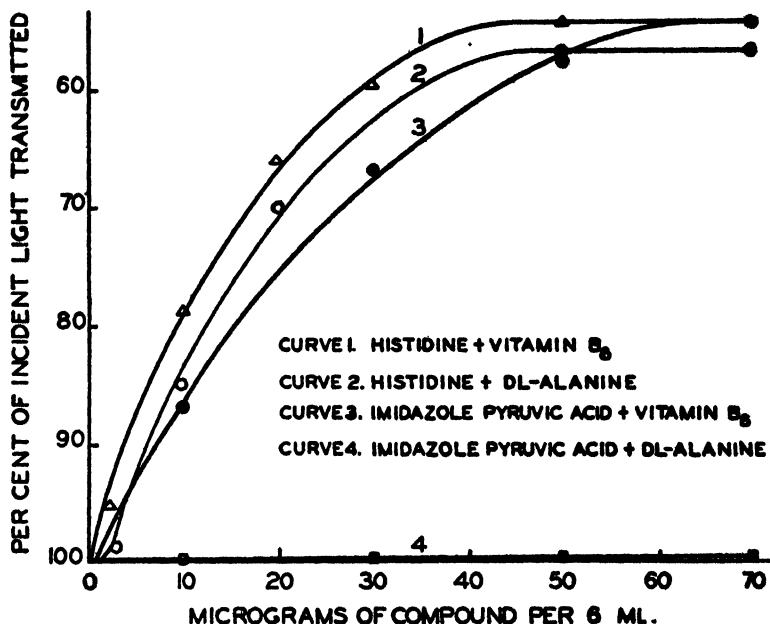
FIG. 5. Comparative activities of histidine and of β-imidazolepyruvic acid in promoting growth of *S faecalis* in the presence and absence of vitamin B_6. Curves 1 and 3, 10 γ of pyridoxal·HCl added per 6 ml.; Curves 2 and 4, pyridoxal omitted and 5 mg. of DL-alanine added per 6 ml. Incubated 24 hours at 37°.

convincing evidence for the essential rôle of vitamin B_6 in the conversion of this keto acid to the amino acid.

DISCUSSION

Results of the present study are in full accord with those of recent tracer experiments (3–5) and indicate that in these bacteria, as in animals, histidine does not serve as a precursor of purine bases. The finding that purine bases do apparently serve as precursors for synthesis of histidine should not be considered contradictory to the report of Cox and Rose (6),

who found that neither adenine, guanine, nor a combination of these compounds was able to replace histidine for growth of the rat. The latter animal, like *S. faecalis*, requires histidine for growth even though purine bases are present in the ration; *i.e.*, the step in the synthesis of histidine which is deficient in these organisms lies between the purine bases and histidine (but before imidazolepyruvic acid) and consequently addition of the purine bases does not permit growth. In *L. arabinosus*, by contrast, the rate at which appropriate purine bases are synthesized limits the rate at which histidine can be synthesized; consequently, addition of either histidine or purine bases greatly enhances the rate of growth. Since purine bases are also required for purposes other than histidine synthesis, the further addition of purine bases to a medium containing histidine further enhances the growth rate (Fig. 4). Since the function served by the purine bases in the absence of histidine differs from that when histidine is present, it is easily seen why one purine base (xanthine) should be superior to another (hypoxanthine) in the absence of histidine, and how these relationships might be changed when histidine was present.

The finding that vitamin B_6 is essential for conversion of imidazolepyruvic acid to histidine is of considerable interest, since it implies far wider significance for the transamination reaction in living organisms than has been indicated by the results of enzyme studies *in vitro* (24–26).

SUMMARY

In a synthetic medium free of purine bases and histidine, addition of *either* purine bases *or* histidine greatly stimulates growth of *Lactobacillus arabinosus*. This indicates a metabolic relationship between these compounds. If vitamin B_6 is omitted from the medium, only histidine is effective in promoting growth.

The histidine requirement of *Streptococcus faecalis* was identical whether this organism was grown under conditions such that it synthesized purine bases, or whether it was grown under conditions such that it required purine bases preformed in the medium. It was concluded, therefore, that histidine does not serve as a precursor for synthesis of purine bases. In contrast, the requirement of *Lactobacillus casei* for purine bases was enhanced when this organism was grown in the absence of histidine. The conclusions were drawn that the purine bases serve as precursors for histidine synthesis in these organisms, and that vitamin B_6 is involved in this synthesis.

Imidazolealdehyde inhibits growth of *L. arabinosus;* this inhibition is partially overcome by the individual addition of either purine bases or histidine, and is completely eliminated by a mixture of the two. The data are consistent with the interpretation that purine bases serve as precursors for histidine synthesis in these bacteria.

Imidazolepyruvic acid replaces histidine for both *L. arabinosus* and *S. faecalis* when vitamin B$_6$ is present, but not when the vitamin is absent. It is postulated that the keto acid is a normal intermediate in histidine synthesis, and that vitamin B$_6$ is required for its conversion to histidine, probably by transamination. Imidazoleacrylic and imidazolelactic acids did not substitute for histidine under any conditions, and are probably not normal intermediates in histidine synthesis in these bacteria.

BIBLIOGRAPHY

1. Ackroyd, H., and Hopkins, F. G., *Biochem. J.*, **10**, 551 (1946).
2. Rose, W. C., and Cook, K. G., *J. Biol. Chem.*, **64**, 325 (1925).
3. Barnes, F. W., Jr., and Schoenheimer, R., *J. Biol. Chem.*, **151**, 123 (1943).
4. Tesar, C., and Rittenberg, D., *J. Biol. Chem.*, **170**, 35 (1947).
5. Sonne, J. C., Buchanan, J. M., and Delluva, A. M., *J. Biol. Chem.*, **173**, 69 (1948).
6. Cox, G. J., and Rose, W. C., *J. Biol. Chem.*, **68**, 769 (1926).
7. Williams, W. L., Broquist, H. P., and Snell, E. E., *J. Biol. Chem.*, **170**, 619 (1947).
8. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
9. Rabinowitz, J. C., and Snell, E. E., *J. Biol. Chem.*, **169**, 631 (1947).
10. Pyman, L. F., *J. Chem. Soc.*, 186 (1916).
11. Barger, G., and Stewart, C. P., *J. Pharmacol. and Exp. Therap.*, **29**, 223 (1926).
12. Stumpf, P. K., and Green, D. E., *J. Biol. Chem.*, **153**, 387 (1944).
13. Lyman, C. M., Moseley, O., Wood, S., Butler, B., and Hale, F., *J. Biol. Chem.*, **167**, 177 (1947).
14. Snell, E. E., and Mitchell, H. K., *Proc. Nat. Acad. Sc.*, **27**, 1 (1941).
15. Stokes, J. L., *J. Bact.*, **48**, 201 (1944).
16. Rogers, L. L., and Shive, W., *J. Biol. Chem.*, **172**, 751 (1948).
17. Hutchings, B. L., and Peterson, W. H., *Proc. Soc. Exp. Biol. and Med.*, **52**, 36 (1943).
18. Shive, W., and Macow, J., *J. Biol. Chem.*, **162**, 451 (1946).
19. Shive, W., Ackermann, W. W., Gordon, M., Getzendaner, M. E., and Eakin, R. E., *J. Am. Chem. Soc.*, **69**, 725 (1947).
20. Snell, E. E., and Mitchell, H. K., *Arch. Biochem.*, **1**, 93 (1942–43).
21. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, **170**, 133 (1947).
22. Snell, E. E., and Guirard, B. M., *Proc. Nat. Acad. Sc.*, **29**, 66 (1943).
23. Lichstein, H. C, Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, **161**, 311 (1945).
24. O'Kane, D. E., and Gunsalus, I. C., *J. Biol. Chem.*, **170**, 425 (1947).
25. Cohen, P. P., *Biochem. J.*, **33**, 1478 (1939).
26. Green, D. E., Leloir, L. F., and Nocito, V., *J. Biol. Chem.*, **161**, 559 (1945).

ELECTROPHORESIS AND SOLUBILITY OF CHYMOTRYPSINOGEN B AND CHYMOTRYPSIN B*

By VIRGINIA KUBACKI,† KENNETH D. BROWN,‡
AND M. LASKOWSKI

(*From the Department of Biochemistry, Marquette University
School of Medicine, Milwaukee*)

During the past few years the work carried out in this laboratory on proteins from beef pancreas first led to the crystallization of a new protein (2). A year later this protein was identified as a proteolytic zymogen (3). Last year the active enzyme was also obtained in a crystalline form (4). Since the active enzyme resembled chymotrypsins[1] (5, 6) in several respects (4, 7), the name chymotrypsin B was suggested for the active enzyme and chymotrypsinogen B for its zymogen (4).

Fruton (7) investigated the specificity of chymotrypsin B toward several synthetic substrates and found it to be identical with that of other chymotrypsins. The differences in chemical properties were, however, indicated by the method of preparation and by a somewhat slower rate of hydrolysis of casein by chymotrypsin B (4).

The purpose of the work presented in this paper was to investigate the purity of proteins of the B series, and to compare some properties of these proteins with the respective properties of chymotrypsin α and its zymogen.

[1] When originally crystallized by Kunitz and Northrop (5), chymotrypsin had no additional symbol assigned to it for the further identification. Later Kunitz (6) described the crystallization of two other forms, chymotrypsin β and chymotrypsin γ, and referred to the original form as chymotrypsin α. Since at that time only one chymotrypsinogen was known, it was not further specified by a Greek letter. It seems to us that by implication the original chymotrypsinogen should have the symbol α reserved for it. For the sake of brevity and uniformity we refer to both original proteins obtained by Kunitz and Northrop as proteins of the α series, hoping that in so doing we comply with the authors' intention.

EXPERIMENTAL

Chymotrypsinogen B and chymotrypsin B were prepared as previously described (2, 3) and were recrystallized a minimum of four times. Chymotrypsinogen α and chymotrypsin α were prepared according to Kunitz and Northrop (5).

The electrophoretic experiments were conducted as follows. Sufficient protein was dissolved in 20 ml. of buffer of 0.1 ionic strength to make a solution of 1 per cent in respect to protein. Two sets of buffers were used: acetate and glycine-sodium hydroxide. The protein solution was dialyzed at 5° against 1500 ml. of the same buffer for a minimum of 24 hours. Electrophoresis was carried out in a standard 15 ml. cell at 3°. Time, pH, and
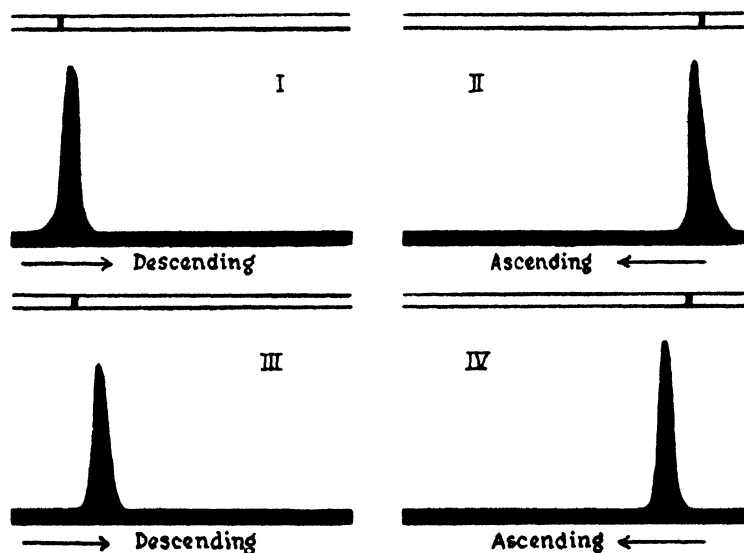
Fig. 1. I and II, patterns of a 1 per cent solution of chymotrypsinogen α in a 0.1 μ glycine-sodium hydroxide buffer at pH 8.44 after 100 minutes at 4.6 volts per cm. III and IV, electrophoretic patterns of a 1 per cent solution of chymotrypsinogen B in a 0.1 μ acetate buffer at pH 5.66 after 100 minutes at 6.76 volts per cm.

field strength are specified for each experiment. Calculations were made from the descending boundary only.

Fig. 1 shows the electrophoretic pattern of chymotrypsinogen B (III and IV). For the purpose of comparison the patterns obtained with chymotrypsinogen α are also shown in Fig. 1 (I and II). Fig. 2 shows the patterns for chymotrypsin α (I and II) and for chymotrypsin B (III and IV). Each of the presented patterns shows a single component, indicat-

ing the "electrophoretic" purity of the protein. No difference in this respect can be found between α and B proteins.

The non-identity of chymotrypsinogen B with chymotrypsinogen α is apparent from Fig. 3. This experiment was performed on lyophilized proteins which were mixed in a 50:50 ratio prior to electrophoresis. The pattern shows the presence of two components in approximately equal amounts, if allowance is made for the small amounts of salt present in lyophilized material. At pH 6.1 the two proteins moved in the opposite direction as shown from their positions relative to the δ and ε boundaries.
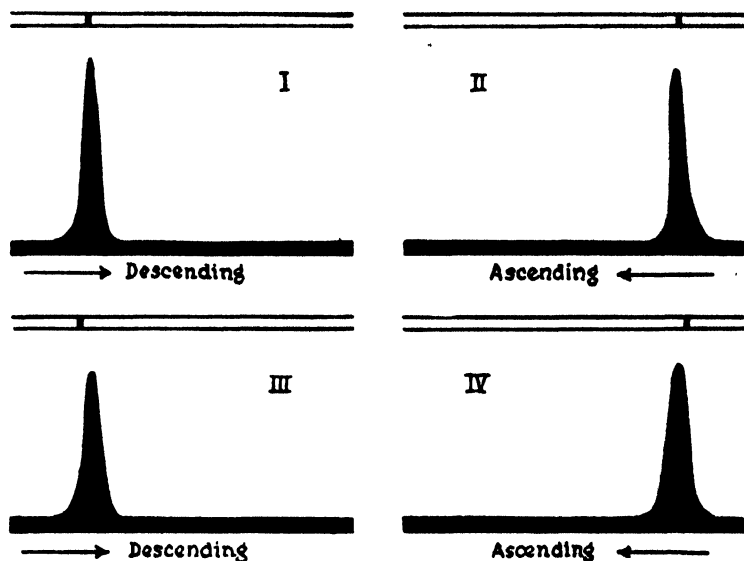
Fig. 2. *I* and *II* are electrophoretic patterns of a 1 per cent solution of chymotrypsin α in a 0.1 μ glycine-sodium hydroxide buffer at pH 8.20 after 120 minutes at 4.6 volts per cm. *III* and *IV*, electrophoretic patterns of a 1 per cent solution of chymotrypsin B in a 0.1 μ acetate buffer at pH 5.10 after 100 minutes at 5.8 volts per cm.

Further proof of non-identity is supplied by the pH-mobility curves (Fig. 4). Under our experimental conditions the zero mobility points of chymotrypsinogen α and chymotrypsin α were found to lie far to the alkaline side of the previously reported isoelectric points of 5.0 and 5.4 respectively, as determined by the migration of collodion particles coated with these proteins (5). It is of interest to note that the slope of the curve of chymotrypsinogen α (Fig. 4) at the region of pH 5.5 is very similar to the slope at the region of pH 8.5.

The curves representing active chymotrypsins α and B are similar in shape to the respective curves of their zymogens, but are shifted to the

left.   In other words the zero mobility lies in both cases at a lower pH for the active than for the inactive form.   During the process of preparation of chymotrypsin B and its zymogen, as well as during the studies of solubility, it was noticed that the pH at which minimum solubility was observed varied with the concentration of neutral salts.

Solubility studies on chymotrypsin B and its zymogen were conducted according to the method of Kunitz (8).   The concentration of dissolved
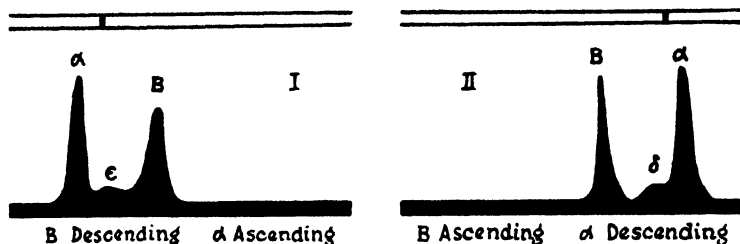
FIG. 3. Electrophoretic pattern of a 1:1 mixture of chymotrypsinogen α and chymotrypsinogen B in a 0.1 μ acetate buffer at pH 6.10 after 100 minutes at 6.7 volts per cm.
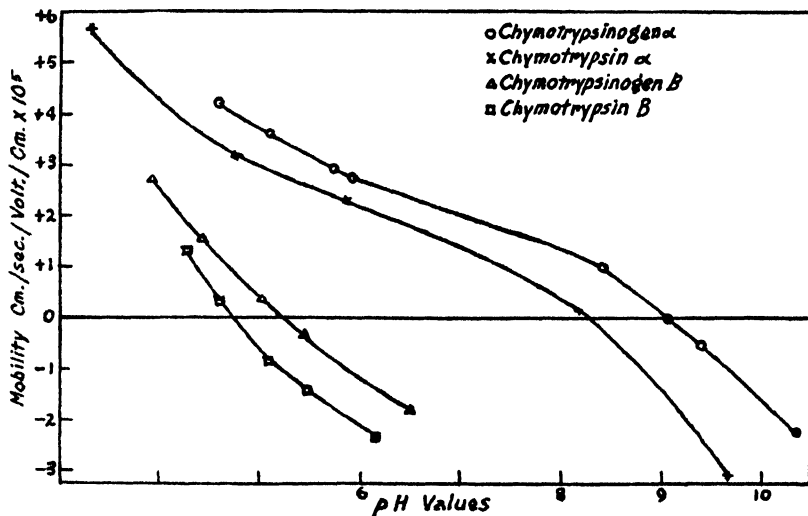
FIG. 4. pH-mobility curves

proteins was determined spectrophotometrically at 280 mμ by using the factors previously reported (4).   Prior to the solubility determination, the proteins were equilibrated with the solvent to be used as many times as was necessary to arrive at a constant concentration in the supernatant liquid.
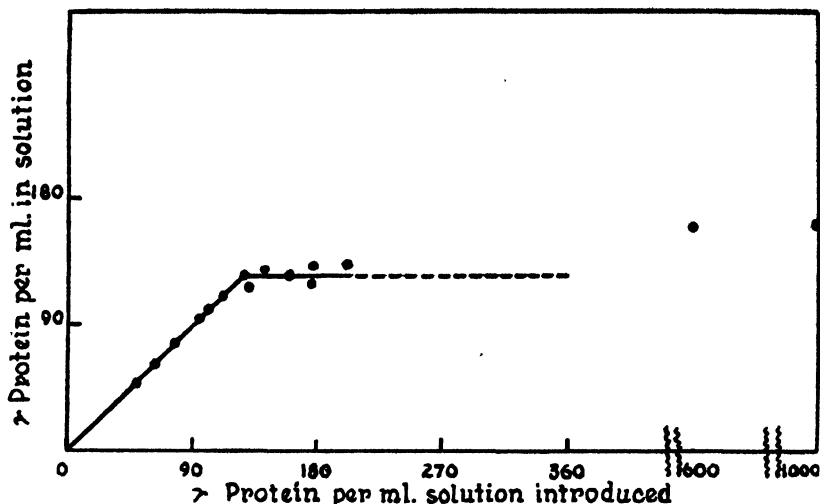
FIG. 5. Solubility curve of chymotrypsinogen B, in 0.1 M acetate buffer, pH 4.97, made 0.27 saturated in respect to ammonium sulfate. The dotted line represents the solubility for a system composed of one component.
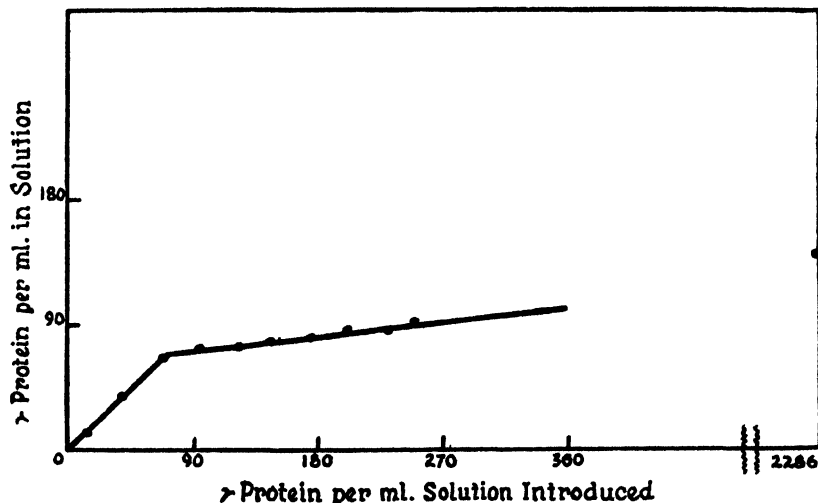
FIG. 6. Solubility curve of chymotrypsin B in 0.1 M acetate buffer, pH 4.08, made 0.5 saturated in respect to ammonium sulfate.

Chymotrypsinogen B used for the solubility studies was prepared according to the method previously described (3) with the exception that each recrystallization was carried out from a larger volume (3 volumes instead of the minimum amount) of solvent. The final recrystallization was made from 0.27 saturated ammonium sulfate.

Fig. 5 shows the experimental results. The dotted line was drawn to represent a system composed of one component. The points obtained with 0.6 and 1.0 mg. of solid phase deviate sufficiently from the theoretical curve to indicate the presence of an impurity. The extent of the deviation indicates that the impurity is present in a relatively small amount.

The material for the determination of solubility of chymotrypsin B was prepared in the usual manner (4). After four recrystallizations chymotrypsin B was dissolved in 10 volumes of 0.5 saturated ammonium sulfate at pH 5.2 and dialyzed against 0.5 saturated ammonium sulfate at pH 4.0 for 48 hours.

The resulting precipitate was amorphous. It was equilibrated with 0.1 M acetate buffer, pH 4.08, made 0.5 saturated in respect to ammonium sulfate. The solubility curve of chymotrypsin B is shown in Fig. 6. The presence of one contaminant is indicated by the shape of the curve. As in the previous case the amount of contaminant is small.

SUMMARY

By the electrophoretic technique chymotrypsinogen B and chymotrypsin B were found to be as pure as chymotrypsinogen α and chymotrypsin α. The zero mobility was found for chymotrypsin B at pH 4.7 and for chymotrypsinogen B at pH 5.2. The B proteins were distinctly different from the respective α proteins. The zero mobility for chymotrypsin α was found at pH 8.3, and for chymotrypsinogen α at pH 9.1.

By the solubility studies the presence of a small amount of contaminant has been demonstrated in both chymotrypsinogen B and chymotrypsin B.

Addendum—After this manuscript was completed, the paper of Anderson and Alberty (9) came to our attention. These authors reported the isoelectric point for chymotrypsinogen α as 9.5 and for chymotrypsin α as 8.6 in 0.01 μ buffers, and 8.1 for chymotrypsin α in 0.1 μ buffer. Our results agree with these values fairly well.

BIBLIOGRAPHY

1. Kubacki, V., Brown, K. D., and Laskowski, M., *Federation Proc.*, **8**, 216 (1949).
2. Laskowski, M., *J. Biol. Chem.*, **166**, 555 (1946).
3. Keith, C. K., Kazenko, A., and Laskowski, M., *J. Biol. Chem.*, **170**, 227 (1947).
4. Brown, K. D., Shupe, R. E., and Laskowski, M., *J. Biol. Chem.*, **173**, 99 (1948).
5. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **18**, 433 (1934–35).
6. Kunitz, M., *J. Gen. Physiol.*, **22**, 207 (1938).
7. Fruton, J. S., *J. Biol. Chem.*, **173**, 109 (1948).
8. Kunitz, M., *J. Gen. Physiol.*, **13**, 788 (1930).
9. Anderson, E. A., and Alberty, R. A., *J. Phys. and Colloid Chem.*, **52**, 1345 (1948).

THE NITROGENOUS METABOLISM OF THE EARTHWORM (LUMBRICUS TERRESTRIS)

By STANLEY COHEN and HOWARD B. LEWIS

(From the Department of Biological Chemistry, Medical School, University of Michigan, Ann Arbor)

Our knowledge of the nitrogenous metabolism of most invertebrates is meager. As a contribution to the study of comparative biochemistry, we have investigated in some detail the excretion of compounds of nitrogen by the earthworm, *Lumbricus terrestris*, a representative of the phylum, Annelida.

In 1908, Lesser (1), after storage of fresh earthworms (*Lumbricus agricola, Allolobophora foetida*) for 24 hours, was unable to detect in the washings uric acid, allantoin, or urea, although traces of ammonia were present. The enzymes, xanthine oxidase, uricase, allantoinase, and allantoicase, are apparently not present in the tissues of *Lumbricus* (2, 3), although the presence of urease in small amounts in *Lumbricus agricola* has been claimed (4). However, urea could not be detected in extracts of the tissue of earthworms (5).

According to Delaunay (6), the nitrogen of the excreta of earthworms consisted largely of ammonia with smaller amounts of urea and amino acid nitrogen, while uric acid was not present; urea accounted for from 6 to 38 per cent of the total nitrogen. After an 8 day period of inanition, the excretion of urea was increased 2- to 4-fold over the initial level. The smaller amounts of urea in the excreta of normal worms were ascribed to the decomposition of some urea by bacteria present in the gut. In the fasted worms, the amounts of solid material (and presumably bacteria) excreted were much smaller. The diminished bacterial activity was believed to be the factor in the higher content of urea in the excreta. In extracts of whole worms, traces only of uric acid were present, while considerable amounts of urea (12 mg. per cent) and ammonia nitrogen (10 mg. per cent) were found.

The most extensive investigation of the metabolism of the earthworm, that of Heidermanns in 1937 (7), showed a much greater content of urea in the intestinal tissue than in the body wall. Urea was formed when peptone was incubated with mince prepared from the whole worm and from the intestine, but not with mince prepared from the body wall. Similarly preparations of intact tissue of the intestine formed urea in a peptone-containing medium. Neither arginine nor ornithine could replace the

peptone; these compounds did not influence the synthesis of urea by the intestinal tissue. When ammonium salts were added, some ammonia disappeared, but no increased production of urea could be demonstrated.

It was concluded that urea was formed in the intestinal wall, presumably by the chlorogogue cells, and excreted by the nephridia, that arginase was not present, and that the ornithine cycle of Krebs and Henseleit did not participate in the synthesis of urea in the earthworm (7).

<div align="center">EXPERIMENTAL</div>

Animals—Mature earthworms (*Lumbricus terrestris*), ranging in weight from 3 to 5 gm. each, were collected on the university campus and were used within 24 hours after collection. No differences in the analytical values which could be related to seasonal differences (March to November) were observed.

Collection of Excreta of Normal Earthworms—A container was designed to facilitate the quantitative collection of excreted material. A 15 cm. length of glass tubing 7 mm. in diameter was fused to a small hole in the outer rim of the bottom of a 250 ml. Erlenmeyer flask. The tubing was inclined downward at a 30° angle from the vertical toward the center of the flask for a distance of 6 cm., and then vertically downward for the remaining distance. A 1-holed rubber stopper was fitted onto the glass tube, thus enabling the flask to be inserted in a 50 ml. centrifuge tube into which the excreta passed. To prevent the worms, which were placed in the flask, from crawling down the glass tube, a rubber-tipped glass rod was inserted into the tube, the rubber tip extending for 2 cm. The flask and rod were covered with a beaker during a collection period.

All the experiments were conducted at a temperature of 8–12°. Three worms, gathered the previous night, were weighed, rinsed with distilled water, and placed in each flask. The worms were washed daily with 5 ml. of distilled water; the washings together with any solid excreta were allowed to drain into the centrifuge tube, which contained several small crystals of thymol. At the end of the 3rd day, the worms were washed with approximately 10 ml. of distilled water, the 50 ml. collection tube was centrifuged to remove the solid dejecta, and the supernatant liquid was poured into a 25 or 50 ml. volumetric flask with the aid of two washings of 2 ml. each of distilled water. The contents of the flask were diluted to the mark with water and aliquots of this solution were used for the analyses. 1 ml. portions were used for the determination of ammonia, urea, and total nitrogen, respectively, and 5 ml. for the determination of allantoin. Aliquots, equivalent to 4.5 and 0.833 ml., were employed for the analyses of uric acid and nonprotein nitrogen respectively.

Feeding Technique and Collection of Excreta in Feeding Experiments—In the feeding experiments, 0.1 ml. portions of the solutions were administered quantitatively with the aid of an automatic syringe of the Krogh and Keys (8) type.[1] A fine glass capillary tube (4.5 cm. in length and 0.9 mm. in outer diameter) was attached to a shortened syringe needle of approximately the same diameter as the capillary tube, by means of fine flexible plastic tubing, 1 cm. in length. The end of the glass capillary had been sealed with the formation of a very slight bulb, and a new opening blown at the side of the tip, approximately 1 mm. from the end. The syringe was then calibrated to deliver 0.1 ml. with an error not exceeding ±0.003 ml.

Partial narcotization of the animals was found necessary for the satisfactory administration of the solutions. Carbon dioxide, which has been used as an anesthetic for insects (9), stupefies earthworms in a few minutes at room temperature.

The compounds to be studied were dissolved so that 0.1 ml. of the solution contained the amount of nitrogen desired. The earthworms were weighed, placed in small Erlenmeyer flasks, and narcotized by allowing a gentle stream of carbon dioxide from a cylinder to flow over them for 3 minutes. The worms should not be completely stunned, but merely slowed in their movements. The animals were then removed, placed on a sheet of filter paper, and gently rolled to remove any adhering mucus. They were then held near the anterior end with the left hand and the capillary tube attached to the syringe was inserted gently into the mouth. The insertion of the tube could be facilitated greatly by stretching the anterior portion of the worm with the fingers of the left hand, in order to straighten the esophagus. The capillary should be inserted into the gizzard to prevent regurgitation. This was accomplished by the insertion of the entire length of the capillary tube (approximately 4.5 cm.). If the worm was not stupefied completely, its own slight movements assisted this operation. The solution was then injected and the capillary tube was removed. The worms were placed on a clean filter paper to observe any regurgitation. This never occurred if the feeding was accomplished correctly. If regurgitation was observed, the animal was discarded. The animals were then rinsed thoroughly with tap water and with distilled water successively. The worms were put into individual 25 ml. Erlenmeyer flasks, and the flasks were covered with beakers and placed in the refrigerator. At the end of 3 days, washings from each of three uninjured worms[2] were combined in a 25 ml. volumetric flask. The washing of each worm was made

---

[1] These syringes are obtainable from the Northern Tool and Die Company, Flushing, New York.

[2] Worms were occasionally injured by a faulty feeding technique or by the administration of excess quantities of nitrogen.

with the aid of four 2 ml. portions of distilled water. The washings were made up to volume and aliquots were used for analysis.

Analytical Methods—Ammonia was determined by the micro diffusion technique of Conway (10), and urea by the urease micro diffusion method of the same author (10). Total nitrogen values were obtained by digestion with a mixture of potassium sulfate and sulfuric acid, followed by nesslerization. Non-protein nitrogen was determined similarly, after the deproteinization of the excreta with trichloroacetic acid (5 per cent). Analysis for uric acid was made by an adaptation of the method of Christman and Ravwitch (11) for use with the Klett-Summerson photoelectric colorimeter and for allantoin plus allantoic acid according to the procedure of Christman, Foster, and Esterer (12).

Preparations Used—L-Ornithine hydrochloride was prepared by the enzymatic hydrolysis of L-arginine according to the method of Hunter (13), L-citrulline from L-ornithine and urea by the method of Gornall and Hunter (14), and hydantoin from hydantoic acid by the procedure of Wagner and Simons (15). Analyses for nitrogen showed that the compounds thus prepared were of satisfactory purity. Glutamine was obtained from Dr. H. B. Vickery and allantoin was obtained from Dr. A. A. Christman. The other amino acids employed were pure commercial preparations. All which contained an asymmetric carbon atom were of the L configuration.

The partition of the nitrogenous materials excreted by fresh[3] and fasted worms was determined. The fasted worms were allowed to remain at a temperature of 8–12° without food for 18 days, with daily removal of excreta, before their nitrogenous excretion was measured. Usually 25 to 100 mg. of solid material were eliminated by a fresh worm over a 3 day period and progressively lesser amounts during prolonged fasting, if coprophagy was prevented so far as possible. The results are shown in Table I.

It can be seen that, while, in fresh worms, from 66 to 91 per cent of the total non-protein nitrogen was excreted as ammonia and less than 10 per cent as urea, in fasted worms, the nitrogen partition was reversed. After 18 days fasting, from 82 to 86 per cent of the nitrogen appeared as urea and less than 10 per cent as ammonia. Upon fasting, there was a 2- to 5-fold increase in the non-protein nitrogen excreted and an even more marked increase in the amount of urea produced.

Even though the analytical methods used were not entirely specific, it may be concluded from Table I that neither uric acid, allantoin, nor allantoic acid was excreted in appreciable amounts by either fresh or fasted earthworms.

[3] The term *fresh* as used in this paper refers to earthworms which had been collected within 24 hours prior to an experimental procedure.

From 71 to 97 per cent of the non-protein nitrogen excreted by fresh worms and 91 to 95 per cent of the non-protein nitrogen by fasted worms, respectively, may be accounted for by the sum of the values obtained in the analyses (last column, Table I). The wide variation in fresh worms is probably due to the differences in the amounts of earthy material excreted.

The absence of urea in the excreta of fresh worms was not due to bacterial decomposition. No disappearance of urea could be detected after

TABLE I

Partition of Nitrogenous Material Excreted by Fresh and Fasted Earthworms

Three worms were used in each experiment; the washings were collected during a 3 day period. The results obtained are calculated as micrograms of nitrogen excreted per 10 gm. of worm per day. The results thus represent the average daily excretion over a 3 day period. The data in Experiments 1 to 4 present the results obtained with fresh worms; the results obtained with worms fasted 18 days prior to the collection of the excreta are recorded in Experiments 5 to 8.

Experiment No.	Nitrogen excreted as					
	NH$_3$	Urea	Uric acid	Allantoin*	Non-protein N	
					Observed	Calculated†
1	52	1	0.3	1.3	57	55
2	82	7	1.2	1.6	105	92
3	56	1	1.2	1.7	85	60
4	40	6	1.8	1.8	61	50
5	23	195	1.9	Trace	231	220
6	15	158	1.2	"	189	174
7	22	237	2.4	"	275	262
8	14	136	1.1	"	166	151

* The allantoin values include any allantoic acid which may be present, since both are determined by the procedure used.

† The calculated non-protein nitrogen values are the summation of the ammonia, urea, uric acid, and allantoin nitrogen values.

the incubation of urea with the intestinal contents of earthworms for 24 hours. Also, when 10 γ of urea were added to three fresh worms in a metabolism flask, 8.6 to 9.4 γ of the urea nitrogen were recovered. Additional experiments indicated that the ammonia present was not derived from the decomposition of nitrogenous material after the excreta had been collected. It is not known whether the ammonia originally present in the intestinal tract was formed by such a decomposition or was excreted by the worms themselves.

The excreta were then collected continuously in 3 day periods for 33 days. The results are summarized in Table II. It is to be noted that

the earthworms were fasted during the entire period of the experiment. Thus, from the almost negligible amounts of urea excreted by fresh worms, the formation of urea increased until, at the end of 33 days of fasting, from 528 to 878 γ of urea nitrogen per 10 gm. of worms per 24

TABLE II

Nitrogenous Excretion by Earthworms during Fasting

Three worms were used in each experiment; the washings were collected in successive 3 day periods up to 33 days. The results are calculated as micrograms of nitrogen excreted per 10 gm. of worm per day, as in Table I. The data shown are the average results for the indicated number of experiments. The values in parentheses indicate the range of the results obtained.

Period No.	Nitrogen excreted as							
	Urea	No. of experiments	NH₃	No. of experiments	Non-protein N	No. of experiments	Total	No. of experiments
1	3 (0– 8)	6	59 (32– 91)	6	96 (75–142)	4	161 (131– 190)	5
2	22 (10– 45)	7	74 (45–113)	7	143 (96–196)	5	221 (144– 288)	4
3	85 (32–120)	5	56 (2– 99)	5	172 (45–288)	3	139 (46– 232)	2
4	121 (110–130)	3	55 (50– 63)	3	200	1	293	1
5	227 (153–275)	3	67 (55– 76)	3	356	1	367	1
6	274 (228–319)	2	31 (29– 33)	2				
7	243 (174–396)	9	26 (4– 98)	9			302 (219– 390)	7
8	372 (213–565)	10	70 (22–128)	10	548 (532–565)	2	552 (351– 756)	8
9	461 (124–906)	7	52 (21–119)	7	487 (475–498)	2	510 (190– 962)	6
10	697 (528–878)	6	48 (25– 63)	6	718 (634–802)	2	860 (658–1130)	5
11	624 (500–738)	6	34 (2– 60)	6			658 (583– 747)	4

hours were being excreted. The rate of urea formation reached a maximum after 24 to 30 days of fasting and then appeared to decrease. Several of the worms died after 33 days and the experiment was discontinued.

The variations in the amounts of ammonia excreted were not due to bacterial action subsequent to collection of the dejecta. A direct cor-

relation between the amount of ammonia present and the quantity of débris excreted was noted, although this relationship was not an exact one. Heidermanns (7) had concluded that most of the ammonia originated in the intestinal tract, though a nephridial origin of part of the ammonia was not excluded. The results obtained support this view.

Considerable amounts of nitrogenous material precipitable by trichloroacetic acid were found in the washings. This might have been expected, since no separation of "urinary" from "fecal" excreta was made. Some mucoid-like material secreted by the surface of the worm was also included. Nevertheless, an increase in the nitrogenous excretion with fasting was noted in both the total and non-protein nitrogen fractions. These results were taken to indicate that tissue protein catabolism increased during fasting, the nitrogenous groups liberated being converted to urea and excreted.

The mechanism of urea formation was studied by a series of experiments in which possible precursors of urea were administered orally and the effect upon the excretion of urea was determined. It seemed preferable to conduct the experiments during inanition, since it was known that the mechanism for urea synthesis was present and operative at this time. However, the changing basal level of urea excretion in fasting made success unlikely in view of the limited amounts of material which could be administered. Therefore, only fresh worms were used in which, due to the constant and almost negligible endogenous urea formation, slight increases in urea excretion might be detected. In all cases, the worms were collected within 24 hours prior to the experiments.

Controls—The total amount of urea nitrogen excreted by three worms during the 3 day period following the administration of distilled water (Table III) varied between 0 and 40 γ, with an average of 17 γ. No essential difference was noted between the quantities of nitrogen excreted by fresh worms fed water and the amounts by normal fresh worms.

Urea—The administration of urea (Table III) resulted in a marked increase in the excretion of urea. Three worms, fed a total of 3000 γ of urea nitrogen, excreted between 540 γ and 800 γ of urea nitrogen (18 to 27 per cent of the nitrogen administered) during the collection period (3 days). Additional amounts of from 540 γ to 760 γ of urea nitrogen were excreted during the 3 day period immediately following the experimental period. The quantities of undetermined nitrogen and ammonia nitrogen in both the animals fed urea and those fed water were approximately equal, which indicated that the nitrogen of the urea administered was not excreted in any form other than as urea and possibly ammonia.

Arginine—The excretion of urea increased as a result of the administration of arginine monohydrochloride. If one disregards the endoge-

TABLE III

Nitrogenous Excretion of Earthworms Following Oral Administration of Nitrogen-Containing Compounds

0.1 ml. portions of solutions containing the desired quantities of the indicated compounds were administered orally to each worm. The wet weight of the three worms in each experiment varied from 9.0 to 13.8 gm. The excreta were collected during the subsequent 3 day period; the washings from three worms were pooled, made up to 25 ml., and analyzed. The results are recorded as micrograms excreted by a single worm in a 3 day period. The data shown are the average results for the indicated number of experiments. The values in parentheses under the average values indicate the range of the results obtained.

Compound administered	As N per worm	Urea	No. of experiments	NH₃	No. of experiments	Non-protein N	No. of experiments
	mg.						
Water	0.00	6 (0– 13)	8	52 (35– 93)	8	103 (59–163)	5
Urea	1.00	230 (180–267)	3	96 (83–103)	3	364 (316–393)	3
Arginine*	2.00	84 (28–132)	7	139 (97–184)	7	560 (440–691)	6
Alanine	1.00	2 (0– 6)	5	42 (9– 88)	5	197 (115–316)	4
Glycine	0.50	5 (3– 11)	8	66 (11– 93)	8		
Histidine	0.50	6 (0– 10)	5	32 (1– 65)	5		
Glutamine	0.50	3 (0– 7)	6	30 (19– 48)	6		
Glutamic acid†	0.25	4 (0– 12)	6	66 (44– 93)	6		
Hydantoin	0.50	6 (2– 12)	5	85 (67–105)	6		
Ammonium chloride	0.20	5 (1– 9)	7	57 (8– 98)	7		
Ornithine*	1.00	4 (0– 8)	4	28 (13– 39)	4	221 (125–303)	3
Citrulline	1.50	17 (3– 36)	7	32 (7– 45)	7	232 (185–270)	6
"	0.50	13 (8– 17)	4	59 (40– 83)	4		

* Arginine and ornithine were administered as the monohydrochloride.

† The glutamic acid was neutralized to pH 7 with NaOH before administration in this and subsequent experiments.

nous formation, from 1.4 to 6.6 per cent of the arginine nitrogen fed was recovered as urea nitrogen. However, if one considers the non-protein nitrogen values, it may be calculated that, of the extra nitrogen which was excreted, 5.0 to 27.2 per cent appeared as urea. If one assumes that only the amidine nitrogens of arginine contribute appreciably to the formation of urea, which seems probable from later experiments with ornithine, the percentage recovery of urea from administered arginine would be even greater.

An average increase in the excretion of ammonia may also be noted. It is not possible to say whether the increased ammonia was derived from the metabolism of arginine by the earthworm or from bacterial action in the intestine. The increases in the undetermined nitrogen present in the non-protein nitrogen values obtained may be accounted for, at least partially, by an excretion of unchanged arginine, since positive Sakaguchi tests were obtained on the deproteinized excreta.

The increased excretion of urea following the ingestion of arginine was not due to bacterial action. The incubation of arginine with intestinal excreta for 3 days at 8–12° showed that, although some ammonia was produced, no significant amount of urea was formed.

Ornithine and Citrulline—The administration of ornithine monohydrochloride did not affect the basal level of urea excretion (Table III). The ingestion of citrulline gave rise to small but significant increases in the amount of urea excreted by fresh worms.

Other Nitrogenous Compounds—The ingestion of alanine did not increase the level of urea excretion over that of the basal. The non-protein nitrogen values indicate that only a fraction (less than 20 per cent) of the ingested nitrogen was excreted in 3 days. Similarly, no effect on the excretion of urea could be detected after the ingestion of glycine, histidine, glutamine, glutamic acid, hydantoin, or ammonium chloride (Table III).

It should be noted that many of these compounds were toxic when administered in too great amounts. A given level of nitrogen administration was arbitrarily considered to be toxic when over half of the worms fed did not survive.

The degree of toxicity of the nitrogenous compounds fed showed considerable variation. Thus alanine and ornithine were toxic at levels of 2 mg. of nitrogen, glycine and histidine at 1 mg. of nitrogen, and ammonium chloride, ammonium citrate, and glutamic acid at 0.5 mg. of nitrogen.

Mixtures of Citrulline and Other, Nitrogenous Compounds—Mixtures of citrulline with other amino acids were fed to see whether the ingestion of a source of nitrogen simultaneously with the citrulline would increase

the production of urea. The excretion of urea following the administration of citrulline together with glycine was ·greater, in most instances, than the sum of the urea produced subsequent to the ingestion of citrulline and glycine singly (Table IV). Glycine was not specific in this respect, since mixtures of citrulline and alanine, and of citrulline, alanine, and glutamic acid, when administered to earthworms, increased the excre-

TABLE IV

Nitrogenous Excretion of Earthworms Following Oral Administration of Mixtures of Nitrogen-Containing Compounds

0.1 ml. portions of solutions containing the quantities of the mixture of compounds indicated were administered orally to each worm. The data are recorded as in Table III.

No of experiments	Compound administered		Nitrogen excretion as	
		As N per worm	Urea	NH₃
		mg.	γ	γ
7	Citrulline	1.50	37	98
	Glycine	0.50	(17–50)	(58–144)
3	Citrulline	0.75	33	68
	Alanine	1.00	(25–48)	(56– 81)
2	Citrulline	1.50	38	86
	Alanine	0.50	(35–39)	(46–125)
	Glutamic acid	0.20		
6	Citrulline	0.50	13	72
	Ammonium chloride	0.20	(4–19)	(38–111)
5	Citrulline	0.50	24	73
	Glutamic acid	0.20	(10–42)	(58– 94)
2	Ornithine*	1.00	3	34
	Glycine	0.50	(1– 4)	(12– 56)
5	Ornithine	0.50	11	88
	Glycine	0.50	(5–18)	(74–123)
4	Ornithine	0.50	5	67
	Glycine	0.50	(3– 6)	(24–109)
	Glutamic acid	0.20		

* Ornithine was administered as the monohydrochloride.

tion of urea to approximately the same extent as did citrulline and glycine.

Mixtures of citrulline and ammonium chloride and of citrulline and glutamic acid were administered at a lower level of nitrogen than in previous experiments, due to the relative toxicity of the ammonium ions and the glutamic acid. The ingestion of citrulline and ammonia had but slight effect on the excretion of urea; approximately the same amount ·of urea was excreted as when citrulline alone was ingested. However,

the administration of citrulline plus glutamic acid, equivalent in nitrogen content to the mixture of citrulline and ammonium chloride fed, resulted in a slight increase in urea excretion (Table IV).

Apparently, citrulline and glutamic acid, when administered orally, are more efficiently utilized for the synthesis of urea than an equivalent amount (in terms of nitrogen) of citrulline and ammonium chloride.

No definite conclusion can be drawn from the data concerning the relative efficiencies of glycine, alanine, and glutamic acid in increasing the excretion of urea when fed together with citrulline, due partly to the variations in individual experiments, and partly to variations in amounts of nitrogen administered.

Mixtures of Ornithine and Other Nitrogenous Compounds—It was noted previously that the administration of ornithine did not increase the excretion of urea. The simultaneous ingestion of either ornithine and glycine or ornithine, glycine, and glutamic acid similarly had no effect on the quantities of urea eliminated (Table IV).

DISCUSSION

The nitrogenous material excreted by earthworms during the 3 day period immediately following the removal of the animals from their natural environment was in the form of ammonia mainly; traces of uric acid, urea, and possibly allantoin were also detected. In addition, Delaunay (6) has reported that some amino and purine nitrogen were also present in the excreta. The pattern of nitrogenous excretion underwent a marked change on prolonged inanition, characterized by a progressive increase in the quantity of urea synthesized. Whereas initially earthworms excreted urea nitrogen in the order of 0.05 mg. per 100 gm. per 24 hours, worms fasted for 24 to 33 days excreted up to 9.06 mg. of urea nitrogen per 100 gm. in 24 hours. Similarly, the urea nitrogen content of fresh worms amounted to approximately 1 mg. per 100 gm. of body weight, while after 14 days of inanition, the urea nitrogen content had increased to 57 mg. per 100 gm.[4] The increases in urea synthesis were most marked during the later stages of inanition, reaching a maximum between the 24th and 30th days. The urea excretion then decreased somewhat just prior to death. A 5- to 10-fold increase in the amount of total nitrogen excreted was noted as compared with the quantity of nitrogen eliminated by well nourished worms.

These phenomena were interpreted to mean that the metabolism of protein increased during fasting and that the nitrogen of the catabolized protein was converted to urea and excreted. So far as is known to us,

[4] Unpublished data.

the fasted worm excretes more of its nitrogen in the form of urea than any other invertebrate studied.

Similar changes in the distribution of excreted nitrogen of the lung-fish, *Protopterus aethiopicus*, are reported to occur under the stress of changing environmental conditions. The nitrogen of the fasting lung-fish is excreted about equally as urea and ammonia. During estivation, ammonia formation is apparently suspended and urea accumulates to the extent of 1 to 2 per cent of the body weight in 1 year (16).

Prolonged inanition in the dog results in an initial fall in the nitrogenous excretion to a fairly constant, low level, followed just prior to death by an increase in the amount of nitrogen excreted (17). The excretion of urea by the snail, *Helix pomatia*, decreased during fasting (18).

The urea excreted by the fasted earthworm is undoubtedly formed by a synthetic mechanism and not merely by the action of arginase on the arginine derived from tissue protein, since over 80 per cent of the nitrogen excreted by fasted worms is in the form of urea.

Arginine and, to a smaller extent, citrulline, when administered orally to fresh worms, resulted in an increased excretion of urea. The production of urea was not affected by the ingestion of histidine, glutamine, glutamic acid, glycine, alanine, hydantoin, or ammonium chloride. The simultaneous administration of citrulline with certain other amino acids (glycine, alanine, or glutamic acid) led to an increase in the quantity of urea excreted which was greater than the sum of the amounts of urea excreted when citrulline and the amino acids were administered singly. These results may be explained by assuming that citrulline was converted into arginine, and that the efficiency of this conversion was increased by the simultaneous ingestion of a readily available source of nitrogen. However, no evidence was obtained to indicate that ornithine might be converted into arginine also. The oral administration of ornithine, or of mixtures of ornithine and glycine or ornithine, glycine, and glutamic acid, produced no appreciable increase in the excretion of urea. It should be noted that, because of the changing levels of elimination of urea during fasting, it was necessary in these experiments to use well nourished worms in which the amount of endogenous urea excreted is very slight and relatively constant. Thus, evidence has been adduced to indicate that the earthworm is able to convert the nitrogen present in arginine[5] and probably that present in citrulline into urea. No effect of ornithine on the excretion of urea could be detected.

---

[5] Studies of the distribution of arginase in the earthworm and of its properties will be presented in a paper to be submitted presently.

SUMMARY

1. A procedure for the quantitative oral administration of 0.1 ml. portions of a solution to earthworms has been devised.

2. The non-protein nitrogen excreted by the earthworm (*Lumbricus terrestris*) was mainly in the form of ammonia; traces of urea, uric acid, and possibly allantoin were also present. During inanition, the quantity of urea excreted by the animal increased markedly.

3. The oral administration of arginine and, to a smaller extent, of citrulline resulted in an increased excretion of urea. The ingestion of ornithine, glutamic acid, glutamine, glycine, alanine, histidine, hydantoin, or ammonium chloride did not affect the elimination of urea.

4. The amount of urea excreted following the ingestion of citrulline was increased somewhat by the simultaneous administration of citrulline with other amino acids such as glycine, alanine, or glutamic acid. The administration of mixtures of ornithine with the other amino acids had no effect on the excretion of urea.

BIBLIOGRAPHY

1. Lesser, E. J., *Z. Biol.*, **50,** 421 (1907-08).
2. Truszkowski, R., and Chajkinówna, S., *Biochem. J.*, **29,** 2361 (1935).
3. Florkin, M., and Duchateau, G., *Arch. internat. physiol.*, **53,** 267 (1943).
4. Przylecki, St. J., *Arch. internat. physiol.*, **20,** 103 (1922-23).
5. Ackermann, D., and Kutscher, F., *Z. Biol.*, **75,** 315 (1922).
6. Delaunay, H., Recherches biochimiques sur l'excrétion azotée des invertébrés, Thesis, Bordeaux (1927).
7. Heidermanns, C., *Zool. Jahrb., Abt. allgem. Zool. u. Physiol.*, **58, 57** (1937-38).
8. Krogh, A., and Keys, A. B., *J. Chem. Soc.*, 2436 (1931).
9. Williams, C. M., *Science*, **103,** 57 (1946).
10. Conway, E. J., Micro-diffusion analysis and volumetric error, London (1939).
11. Christman, A. A., and Ravvitch, S., *J. Biol. Chem.*, **95,** 115 (1932).
12. Christman, A. A., Foster, P. W., and Esterer, M. B., *J. Biol. Chem.*, **155, 161** (1944).
13. Hunter, A., *Biochem. J.*, **33,** 27 (1939).
14. Gornall, A. G., and Hunter, A., *Biochem. J.*, **33,** 170 (1939).
15. Wagner, E. C., and Simons, J. K., *J. Chem. Education*, **13,** 265 (1936).
16. Smith, H. W., *J. Biol. Chem.*, **88,** 97 (1930).
17. Morgulis, S., Fasting and undernutrition, New York, 126 (1923).
18. Baldwin, E., and Needham, J., *Biochem. J.*, **28,** 1372 (1934).

EFFECT OF OXYTHIAMINE ON BLOOD PYRUVATE-LACTATE RELATIONSHIPS AND THE EXCRETION OF THIAMINE IN RATS*

BY CHARLES E. FROHMAN† AND HARRY G. DAY

(From the Department of Chemistry, Indiana University, Bloomington)

Oxythiamine, 4-methyl-5-(β-hydroxyethyl)-N[(2-methyl-4-hydroxypyrimidyl-(5))methyl]thiazolium chloride, has been reported in preliminary communications to have a toxic effect on mice and to inhibit the action of carp thiaminase (1, 2). These reports imply that the effect is due to an antagonistic action on thiamine, but neither definite statements nor data to that effect have been published. A few other analogues and homologues of thiamine have been definitely shown to be antagonists of that vitamin (3), but the investigations have not been concerned with the effects of the antagonists on the blood pyruvate-lactate relationships, or the establishment of direct evidence for the displacement of thiamine from the body. Owing to the lack of information on these questions it seemed desirable to extend the study of thiamine antagonism, with oxythiamine as the analogue of choice.

EXPERIMENTAL

Preparation of Oxythiamine—The method of Soodak and Cerecedo (1) was used, except that the nitrous acid was passed through the thiamine hydrochloride solution without the aid of slight suction. This change was made because slight suction could not be maintained satisfactorily without occasionally drawing air into the reaction flask. Air causes the formation of oxides of nitrogen which reduce the yield of oxythiamine.

Diets—A stock diet and a thiamine-free diet were used. The former was Purina laboratory chow. The latter was composed of vitamin-test casein 18, glucose (cerelose) 67, salts 4(4), hydrogenated cottonseed oil (Crisco) 8, cod liver oil 2, and a vitamin mixture 1. The latter was composed of pyridoxine hydrochloride 0.05, riboflavin 0.05, nicotinic acid 0.25, calcium pantothenate 0.25, inositol 0.50, p-aminobenzoic acid 0.50, choline chloride 6.0, and glucose 42.6.

Effect of Oxythiamine on Concentration of Pyruvic Acid and Lactic Acid in Blood—The data were collected from normal young adult rats on the stock colony ration. The animals were divided equally in respect to sex and weight. For the blood analyses the rats were killed by decapitation and the blood was collected directly into oxalate-iodoacetate in accordance with the Bueding and Wortis (5) procedure. The Bueding and Wortis (5) method was used for the determination of pyruvic acid. Lactic acid was determined by the method of Barker and Summerson (6). Oxythiamine-treated rats were injected intraperitoneally with 150 γ of the compound in 1 ml. of physiological saline. Thiamine was administered in the same way. In the case of animals given both compounds the oxythiamine and

TABLE I

Pyruvic Acid and Lactic Acid in Blood of Stock Rats Following Intraperitoneal Administration of Different Amounts of Oxythiamine and Thiamine

Time after administration	No. of rats	Series No.	Oxythiamine	Thiamine	Pyruvic acid*			Lactic acid found
					Found	Calculated	Excess†	
hrs.			*γ per rat*	*γ per rat*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
1	8	I	0	0	1.4	1.4	0.0	13.1
	8	I	150	0	1.5	1.6	−0.1	15.1
4	12	II	0	0	1.3	1.2	0.1	11.5
	6	IV	0	75	1.1	1.0	0.1	9.5
	12	II	150	0	5.1	2.7	2.4	42.8
	6	III	150	75	2.9	2.3	0.6	25.9
	6	III	150	150	1.1	1.1	0.0	9.8
	6	IV	150	300	0.8	0.8	0.0	7.6

* Stotz and Bessey (7) formula: $PA_{calc.} = LA_{found}/9.44$, when LA < 20 mg. per cent; $PA_{calc} = 1.60 + 0.0264LA$, when LA > 20 mg. per cent.

† $PA_{excess} = PA_{found} - PA_{calc.}$.

thiamine for one dose were dissolved together in 1 ml. of the saline. Following injection with the oxythiamine or thiamine the animals were fasted until they were sacrificed at the end of 1 hour or 4 hours.

The results are summarized in Table I. Control animals given no oxythiamine or extra thiamine had pyruvic acid and lactic acid levels in the normal range reported by Stotz and Bessey (7) and others using good analytical methods. Blood taken 1 hour after the injection of 150 γ of oxythiamine per rat showed no significant rise in either the pyruvic or lactic acid. In contrast, at the end of 4 hours, following oxythiamine administration, marked elevations were found in the blood levels of both metabolites. That this was due to a functional deficiency in thiamine is indicated by the occurrence of pyruvic acid in excess of the amount ex-

pected otherwise on the basis of Stotz and Bessey's (7) data. As shown by these investigators, the blood pyruvic acid level is proportional to the lactic acid in various conditions not associated with thiamine deficiency, e.g. anoxia, excitement, exercise, etc.; but in thiamine deficiency the pyruvic acid to lactic acid ratio is increased.

That the effect of the oxythiamine is due to the production of a functional deficiency in thiamine is indicated further by the results following the administration of thiamine with the oxythiamine. When 75 γ of thiamine were injected with 150 γ of oxythiamine, there was some elevation of both the pyruvic acid and the lactic acid. However, the pyruvic acid excess was only one-fourth as great as in the case of rats given the oxythiamine alone. Amounts of thiamine equal to or double the quantity of oxythiamine given resulted in pyruvic acid and lactic acid levels even less than those in controls receiving no oxythiamine. The pyruvic acid to lactic acid ratios were exactly as predicted by the Stotz and Bessey (7) equations, indicating that the thiamine had completely reversed the anti-thiamine effect of the oxythiamine.

Effect of Oxythiamine on Excretion of Thiamine in Urine—Six litter mate rats, each weighing approximately 100 gm., were divided into two equal groups, placed in individual metabolism cages, and restricted to the thiamine-free diet. Two times a day each rat was given orally 12 γ of thiamine hydrochloride in a small volume of water, making a total of 24 γ per day. The urine of each animal was collected daily for 58 days and analyzed for free thiamine by the thiochrome method of Perlzweig *et al.* (8). After 3 days each of the three rats in Group A was given an intraperitoneal injection of 50 γ of oxythiamine in 1 ml. of physiological saline. This was followed on the 31st day with a similar injection of oxythiamine. Group B rats were given similar injections of oxythiamine on the 19th and 49th days respectively. Each time the animals in one group were given a dose of oxythiamine the animals in the other group were given injections of saline alone.

The analytical results are given in Fig. 1. The data show that animals on the constant thiamine intake excrete rather constant amounts of the vitamin in the urine, approximately 12 γ per rat per day. However, the output of the free thiamine was promptly raised 100 to 175 per cent within 1 to 2 days following the administration of oxythiamine. The high excretion rate subsided to the previous base level, or dropped slightly below it temporarily, after approximately 1 week. Thereafter the level of urinary thiamine excretion remained almost constant until oxythiamine was re-administered. This was followed by another prompt rise in the thiamine excretion, but the amount excreted this time was less than at the first treatment with oxythiamine.

In all cases the amount of extra thiamine excreted following the injection of oxythiamine was as great or greater than the amount of oxythiamine administered. Because preliminary experiments showed that oxythiamine does not interfere in the thiochrome method for determining thiamine, it must be concluded that the high values for thiamine are real, and that an effect of the oxythiamine is the displacement of thiamine from the tissues. The elevated values for thiamine could not have been due to a metabolic transformation of the oxythiamine to thiamine, because the animals exhibited symptoms of acute thiamine deficiency while the large amounts of thiamine were being excreted.

Determinations of thiamine in the tissues were not made, although such data would be useful in interpreting the action of the oxythiamine. In
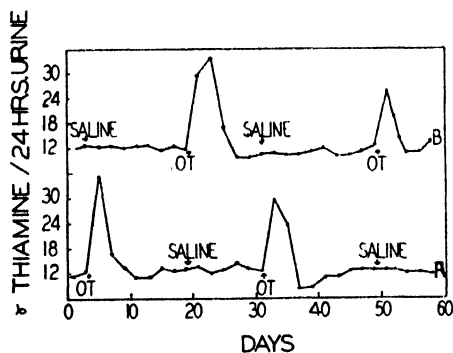
FIG. 1. Effect of oxythiamine (50 γ doses) on the excretion of thiamine in the urine of rats. Each curve (A, B) represents the averaged data from three rats. The arrows indicate the time oxythiamine or saline alone was injected.

this regard it is of value to consider the extent of the thiamine displacement from the tissues. An analysis of the data in Fig. 1 shows that at least 50 γ of thiamine must have been displaced by each 50 γ dose of oxythiamine. Because there probably were not more than 400 γ of thiamine in the tissues (9), it may be estimated that one-eighth or more of the total was displaced. This does not include the displaced thiamine which was probably destroyed.

Gross Effects of Oxythiamine—Another indication of the marked thiamine-displacing action of oxythiamine comes from a study of the gross behavior of the animals. The oxythiamine-treated rats were on the thiamine-free diet but each received 24 γ of the vitamin daily by mouth, an amount which permits considerable thiamine excretion in the urine (Fig. 1) and which is regarded as adequate for rats (10).

The rats became obviously nervous 6 hours after the intraperitoneal in-

jection of 50 γ of oxythiamine. By the end of 12 hours the rats were extremely nervous. Even without any apparent external stimulus they would occasionally jump violently as though they had been shocked with an electric current. Incoordination was manifested. There was evidence of a high degree of generalized cutaneous sensitivity because the rats frequently scratched themselves furiously. No particular part of the body seemed to be favored in this behavior. The apparent itching sensation and irritability gradually subsided in the course of 4 to 6 days. This coincided with the return to normal in the rate of thiamine excretion. We have never observed such extreme irritability or evidence of an itching sensation in rats made thiamine-deficient by restriction to a thiamine-free diet.

DISCUSSION

The data presented here offer direct evidence for the displacement of thiamine from the body by a structural analogue of this vitamin. Hitherto the evidence, in animals, for competition between thiamine and a structural analogue has rested on the effect of thiamine in reversing the symptoms of thiamine deficiency caused by the administration of the analogue. Thus the data support the hypothesis (3) that antimetabolites may act by displacing related metabolites from the specific positions on enzymes and related proteins with which they are reversibly combined.

Another point of interest is the high potency of oxythiamine as an antithiamine compound. As little as 50 γ of the analogue was sufficient to produce symptoms of acute thiamine deficiency and the excretion of considerable amounts of thiamine, even though the animals were receiving approximately 24 γ of thiamine per day. Woolley and White (11) reported that at least 20 γ of pyrithiamine per day were necessary to produce symptoms of thiamine deficiency in mice receiving 2 γ of thiamine per day. Emerson and Southwick (12) found that approximately 40 moles of the 2-n-butylpyrimidine homologue of thiamine produced only slight indications of thiamine deficiency in rats when 1 mole of thiamine was given. Although the dosage levels and the experimental conditions are quite divergent in these different investigations, it is indicated that oxythiamine is a more active antithiamine than either pyrithiamine or the 2-n-butylpyrimidine homologue of thiamine.

SUMMARY

Oxythiamine causes a marked increase in the concentration of blood pyruvic acid and lactic acid of rats. The pyruvic acid to lactic acid ratio is increased as in thiamine deficiency produced with diets low in thiamine. The changes are reversed by thiamine.

The excretion of thiamine in the urine is greatly augmented by oxythiamine. This is interpreted as evidence that the antimetabolite displaces thiamine from the body.

The symptoms of rats suffering from the functional thiamine deficiency include extreme irritability and a tendency for the animals to scratch themselves vigorously.

Oxythiamine is a potent antagonist of thiamine.

BIBLIOGRAPHY

1. Soodak, M., and Cerecedo, L. R., *J. Am. Chem. Soc.*, **66**, 1988 (1944).
2. Soodak, M., and Cerecedo, L. R., *Federation Proc.*, **6**, 293 (1947).
3. Woolley, D. W., *Physiol. Rev.*, **27**, 308 (1947).
4. Jones, J. H., and Foster, C., *J. Nutr.*, **24**, 245 (1942).
5. Bueding, E., and Wortis, H., *J. Biol. Chem.*, **133**, 585 (1940).
6. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, **138**, 535 (1941).
7. Stotz, E., and Bessey, O. A., *J. Biol. Chem.*, **143**, 625 (1942).
8. Perlzweig, W. A., Kamin, H., Gue, I., and Blalock, J. V., *Arch. Biochem.*, **6**, 97 (1945).
9. Sarett, H. P., and Perlzweig, W. A., *J. Nutr.*, **25**, 173 (1943).
10. Schweigert, B. S., McIntire, J. M., and Elvehjem, C. A., *Arch. Biochem.*, **3**, 113 (1943).
11. Woolley, D. W., and White, A. G. C., *J. Biol. Chem.*, **149**, 285 (1943).
12. Emerson, G. A., and Southwick, P. L., *J. Biol. Chem.*, **160**, 169 (1945).

THE OXIDATION AND DISTRIBUTION OF THE METHYL GROUP ADMINISTERED AS METHIONINE*

By COSMO G. MACKENZIE, JOSEPH P. CHANDLER, ELIZABETH B. KELLER, JULIAN R. RACHELE, NANCY CROSS, AND VINCENT DU VIGNEAUD

(From the Department of Biochemistry, Cornell University Medical College, New York City)

It is well known that the methyl radical attached to sulfur or nitrogen in a number of important biological compounds cannot be synthesized by the animal body but must be supplied in the diet. Either methionine, choline, or betaine may serve as the dietary source of the essential methyl group (1). Not only are the methyl groups of these compounds interchangeable in the body, but, directly or indirectly, they provide the methyl groups for the synthesis of still other compounds such as anserine (2), creatine, and creatinine (3). Appreciable quantities of the latter compound are normally excreted in the urine. Thus methyl groups can be lost from the body without undergoing degradation.

Since this essential radical moves *in toto* (3, 4) from compound to compound within the body, and since it can leave the body in an intact form, we wished to ascertain whether the body can oxidize the methyl group to carbon dioxide and water, or whether it is quantitatively excreted in the urine and feces as creatinine and hitherto unidentified methyl compounds. Furthermore, because of the migratory properties of the labile methyl group, it was also of interest to extend our knowledge concerning the rapidity with which the methyl group of administered methionine enters into transmethylation reactions and the speed at which it penetrates into the various tissues and organs of the body.

Oxidation of Methyl Group to Carbon Dioxide

The possibility of obtaining an unequivocal answer to the question concerning the oxidation of the methyl group was provided when Melville, Rachele, and Keller (5), in our laboratory, succeeded in synthesizing L-methionine containing a high concentration of C^{14} in the methyl group.

In our initial experiment 200 mg. of the radioactive methionine were

given by stomach tube to a 165 gm. rat that had previously been fed a purified diet containing 1.2 per cent methionine, 0.2 per cent choline chloride, and no cystine. The labeled methionine was given at 9.00 a.m. and the animal was placed immediately in a metabolism apparatus designed for the continuous and quantitative collection of carbon dioxide. Food was withheld during the remainder of the experiment.

The presence of radioactivity in the carbon dioxide expired during the 1st hour of the experiment demonstrated that the rat was able to oxidize the methyl group administered as methionine. At this time 0.8 per cent of the ingested methyl carbon had appeared in the expired air. The amount of methyl oxidized per hour then rose to a peak value of 2.3 per cent at the 6th and 7th hours. Thereafter the rate of oxidation declined to an average value of 0.29 per cent at the 16th to 24th hours. From this

TABLE I

Oxidation and Excretion of Methyl Carbon of Methionine Expressed As Per Cent of Administered Methionine

Pathway	0-24 hrs.	24-52 hrs.	Total for 52 hrs.
Expired CO_2	26.4	6.0	32.4
Urine	16.2	2.1	18.3*
Feces	0.03	0.4	0.43
Total	42 63	8.5	51.13

* This figure was erroneously given as 14.6 per cent in a preliminary report on the oxidation of the methyl group of methionine (6).

time until the termination of the experiment at 52 hours, the rate of oxidation was approximately constant at 0.23 per cent per hour.

At the end of 52 hours 32.4 per cent of the methyl groups administered as methionine had been completely oxidized to carbon dioxide and water, as indicated by the radioactive carbon dioxide present in the expired air. (This figure does not take into account the carbon dioxide that was formed from methyl groups and subsequently entered into synthetic reactions in the body.) An additional 18.7 per cent of the administered methyl groups was eliminated in the urine and feces either as methyl compounds or as oxidation products of methyl groups. The amount of methyl carbon accounted for in the expired carbon dioxide, urine, and feces on each day of the experiment is shown in Table I. A total of 51 per cent of the administered methyl carbon had left the body by these three routes during the course of the experiment. We next attempted to account for the remaining 49 per cent of the administered methyl carbon.

Anatomical and Chemical Distribution of Methyl Carbon

The intense oxidation of the methyl group during the experiment was accompanied by the ubiquitous appearance of C^{14} in the organs, tissues, and major chemical fractions of the body and by its entry in high concentrations into the newly synthesized creatine and choline. Analysis of fifteen tissues and organs at the end of the experiment revealed the presence of C^{14} in each of them. Although the oxidation to carbon dioxide of the methyl group (administered as methionine) had been constant for 28 hours, the concentration of the methyl carbon in these tissues and organs was far from uniform.

The highest concentrations of the methyl carbon were found in the kidneys, liver, and adrenals. Skeletal muscle and integument, although containing approximately 40 per cent of the total C^{14} present in the body, had relatively low concentrations, and brain, despite its high choline content, possessed the lowest concentration of any tissue, with the exception of the depot fat.

Since, as will be shown below, a considerable proportion of C^{14} in the body was present in methyl compounds, the values obtained for C^{14} concentration must reflect to some extent the concentrations of the administered methyl group in the various tissues and organs.

Following the removal of the small samples (approximately 70 mg. each) of organs and tissues for C^{14} determination, the remainder of the animal, less the blood and kidneys, was frozen in dry ice, finely ground, and subjected to solvent fractionation. Each of the resulting chemical fractions, water-soluble, fat-soluble, trichloroacetic acid-soluble, acetone-soluble, and crude protein, contained appreciable quantities of C^{14}. By far the highest concentration of methyl carbon was found in the water-soluble fraction. 78 per cent of the radioactivity present in this fraction was attributable to choline and creatine. 82 per cent of the radioactivity of the ether-soluble fraction was attributable to choline.

Following the isolation of choline from the ether-soluble material, the remainder of this fraction was further subdivided. The free fatty acids were only slightly radioactive. The cholesterol fraction, of particular interest because of the carbon-bound angular methyl groups, possessed a higher degree of radioactivity. However, analysis of the data showed that but 1 carbon atom in every 800 molecules of cholesterol was derived from the methyl carbon of the administered methionine. A similar degree of radioactivity was observed in the "wax" and "oil" fractions comprising the balance of the unsaponifiable material. Neither of these fractions nor the cholesterol fraction contributed significantly to the total radioactivity of the ether-soluble material.

Over-All Recovery of Methyl Carbon

The per cent of the administered methyl carbon recovered in this experiment may be calculated both from the results of the tissue and organ analyses, and the results of the chemical analyses. Estimation, on the basis of tissue and organ analyses, of the portion of the originally introduced methyl carbon retained in the rat's body gave a value of 101 mg., in terms of methionine, or 50.5 per cent of the original dose. This value taken in conjunction with the per cent of methyl carbon lost from the body in the urine, feces, and expired air (Table I) gives a total recovery of 101.6 per cent.

Estimation of the methyl carbon still present in the body, on the basis of chemical analyses (plus the C^{14} contained in the kidneys and blood), gave a value of 85.6 mg., in terms of methionine, or 42.8 per cent of the original dose. This is a minimum figure, since some loss of radioactive carbon present as carbonate and bicarbonate must have occurred during the trichloroacetic acid extraction. Nevertheless, the total recovery of methyl carbon calculated with this value is 94 per cent.

Participation of Administered Methyl Group in Transmethylation Reactions

37 per cent of the administered methyl carbon still present in the body at 52 hours had participated in transmethylation reactions and was present in the methyl groups of creatine and choline. The high degree of transmethylation activity of the methyl group of the administered methionine reflected by this value is appreciated when one considers that at the same time the crude protein fraction contained only 40 per cent of the administered methyl carbon still present in the body.[1] Equally illuminating in an appraisal of the transmethylation reactions involving the administered methionine molecules is an examination of the specific radioactivity of the creatine, choline, and protein-bound methionine present in the rat after 52 hours of fasting.

In the total body creatine, 1.74 per cent of the methyl groups was derived from the administered methionine. Since approximately 4 per cent of the body creatine is synthesized in 2 days (7), and since all of the radioactive creatine must have been contained in this fraction, 44 per cent of the *newly synthesized creatine* was formed from the administered methionine, although the latter, immediately after its absorption, comprised at the most only 22 per cent of the total body methionine.[2] The proportionately

---

[1] In view of the amount of methyl carbon oxidized to carbon dioxide, it is probable that part of the C^{14} present in the protein fraction was derived from carbon dioxide fixation and was no longer present in the methyl groups of methionine.

[2] The total body methionine is the sum of the administered methionine and the methionine already present in the rat at the beginning of the experiment. The latter

greater contribution of administered methionine to creatine synthesis, as compared with the methionine present in the body protein at the beginning of the experiment, is thus apparent.

Choline was isolated separately from both the water- and ether-soluble fractions. The water-soluble choline was decidedly the more radioactive of the two preparations, in conformity with previous results on rabbits obtained with deuterium-labeled methionine (2). In the present experiment 16.7 per cent of the water-soluble (free) choline molecules and 10.9 per cent of the ether-soluble (phospholipide) choline molecules had derived a methyl group from the administered methionine.

It is interesting to compare the concentrations of administered methyl groups in the two choline fractions with the concentration of labeled methyl groups in protein-bound methionine. According to the compilation of Block and Bolling (8), rat protein contains 3 per cent methionine. If it is assumed that all of the radioactivity in the crude protein fraction is resident in the methyl group of methionine (an exaggerated estimate, as has been previously mentioned), then 5.7 per cent of the protein-bound methionine molecules contained a methyl group supplied by the administered methionine. This is a lower figure than obtains for either of the choline fractions. Accordingly, transmethylation to form choline was a more active process than incorporation of the administered methyl into the total body protein, either by direct introduction of the whole methionine molecule, or by transmethylation between the administered methionine and protein-bound methionine (or homocysteine).

The over-all picture of the metabolism of the methyl group of the 200 mg. of methionine administered in this experiment is one of active participation in both degradative (oxidative) and synthetic (transmethylation) reactions. The rapid rise and fall in the rate of oxidation of the administered methyl group must have reflected a similar rise and fall in the concentration of the radiomethyl in the tissues and cells which oxidize it most rapidly.[3] Although the process of oxidation in itself would lower the concentration, it appears from the wide-spread chemical and morphological distribution of the radiomethyl group observed at the end of the experiment that this was not the only factor involved, but that the chemical and anatomical translocations of the methyl group also contributed to the establishment at 16 to 24 hours of the steady rate of oxidation. Conversely, the establishment of a steady rate of oxidation suggests that the administered

---

was calculated on the assumption that protein made up 14 per cent of the body weight and that 3 per cent of the protein was methionine (8).

[3]Preliminary experiments *in vitro* have shown that the methyl group of labeled methionine is oxidized by liver and kidneys but not by heart or testes.

methyl group, and its oxidation products, had reached at that time an equilibrium with their counterparts already present in the body, and that the assimilation of the radiomethyl group had thus been completed.

The demonstration of the oxidation of the labile methyl group administered as methionine raises a number of important questions, such as the relation between the rate of oxidation and the amount of dietary methionine ingested, the influence of dietary choline and cystine on the oxidation of the methyl group of methionine, and the relative rate of oxidation of the methyl group administered as choline, betaine, sarcosine, creatine, etc. Moreover, the identification of the intermediary products in the oxidation of the methyl groups to carbon dioxide and the determination of their rôles in metabolic processes, as well as the location of the enzyme systems effecting these oxidations, become matters of considerable interest. These and related problems are now under investigation.

<div align="center">EXPERIMENTAL</div>

Collection of Expired Carbon Dioxide—The metabolism apparatus used for the collection of CO_2 and illustrated in Fig. 1 was sturdy, simple, and readily assembled. Rubber tubing was used to connect the members of the assembly. All connections and joints, with the exception of the desiccator stopper and lid, were sealed with glycerol. The former was sealed with beeswax and the latter with Lubriseal.

The flow through the metabolism chamber was 1.1 liters per minute. The pressure within the chamber was approximately 8 mm. of Hg below atmospheric pressure. Except in hot summer weather, the internal temperature of the metabolism chamber, with a rat present, was generally 28° with occasional 1° fluctuations. In hot weather the chamber was held at 28° by draping it with wet towels. A fan placed at variable distances from the desiccator was directed toward it to increase evaporation.

Outward leaks from the apparatus were prevented by the slight negative pressure in the metabolism chamber and the higher negative pressure in the NaOH absorbers. The apparatus was checked for inward leaks by running it for several hours without a rat and with water replacing the alkali in the NaOH scrubbers. If leaks are present, the terminal $Ba(OH)_2$ solution will become cloudy.

During an experiment complete recovery of expired CO_2 was indicated by the absence of cloudiness in the terminal $Ba(OH)_2$ solution. This was the case after 12 hours of continuous operation without replacing the NaOH solution.

In metabolism experiments a second pair of scrubbers containing 2.5 N NaOH was connected to the 3-way stop-cocks shown in Fig. 1. By

switching from one pair of absorbers to the other, collections were made at any desired interval without the loss of expired CO_2.

The 400 ml. of 2.5 N NaOH contained in each tower were prepared from concentrated NaOH and air-free water. Following a collection, the contents of each of the two towers were poured into a 1 liter volumetric flask, each tower was rinsed with two 45 ml. portions of air-free water, and the combined solutions were then made up to volume.

Determination of Radioactivity—Aliquots of the solutions containing Na_2CO_3 were chosen to yield approximately 50 mg. of $BaCO_3$ (30 to 60
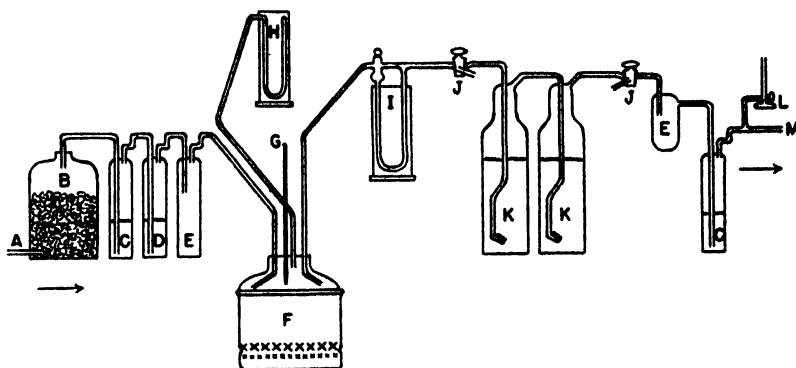
Fig. 1. Metabolism apparatus for collecting expired carbon dioxide. *A*, air inlet tube; *B*, jar of moist NaOH shells (Uehling Instrument Company, Paterson, New Jersey); *C*, 0.4 M $Ba(OH)_2$ solution; *D*, concentrated H_2SO_4 or salt solution for humidity regulation; *E*, trap; *F*, metabolism jar equipped with coarse screen support for rat, and fine screen for collection of feces (the bottom is covered with dilute H_2SO_4 to preserve the urine); *G*, thermometer; *H*, manometer; *I*, flowmeter; *J*, 3-way stop-cock; *K*, 500 ml., coarse porosity fritted disk or cylinder, gas washing bottle containing 400 ml. of 2.5 N NaOH; *L*, Bunsen burner needle valve to regulate air flow through apparatus; *M*, tube to pump.

mg.). Such an aliquot was transferred to a 50 ml. Erlenmeyer flask, brought to approximately 25 ml. with air-free water, stoppered with a CO_2 absorbing tube, and heated to boiling on a hot plate. 2 ml. of 1 M $BaCl_2$ were then introduced rapidly and the flask was immersed in a boiling water bath. This temperature insured the formation of a coarse crystalline precipitate of $BaCO_3$.

The flask was cooled, and the precipitate, following the addition of 5 drops of a 1 per cent solution of Triton NE,[4] was collected on a filter paper

[4] A non-ionic wetting agent obtainable from Rohm and Haas Company, Philadelphia, Pennsylvania.

disk of 2.32 sq. cm. area by the filtration method described by Henriques *et al.* (9) for benzidine sulfate. During the filtration the Erlenmeyer flask was washed three times with 10 ml. portions of water. The collected precipitate was kept covered with water until the last of the washings had been poured into the cylinder. After the last portion of wash water had been sucked through, 4 ml. of absolute alcohol were poured immediately over the precipitate, the cylinder was removed, and the precipitate and filter paper were wetted down with several drops of alcohol. The $BaCO_3$ disk was allowed to dry for 5 minutes at the pump under an infra-red lamp.

The CO_2 evolved from the combustion of tissues, chemical fractions, and compounds obtained from the rat was converted to $BaCO_3$ and made into disks by the method described above. The tissues and chemical preparations were oxidized in a combustion tube, through which there flowed a continuous stream of CO_2-free O_2, and the evolved CO_2 was collected in carbonate-free 2.5 N NaOH.[5] This combustion method has been described by Keller, Rachele, and du Vigneaud (4).

The radioactivity of the $BaCO_3$ samples was measured with the use of a bell-shaped Geiger-Müller counter with a mica window of less than 2 mg. per sq. cm. and an Autoscaler scaling circuit. Samples derived from the expired CO_2, chemical preparations, and tissues were counted alternately with a standard made from the combustion of a weighed amount of the radioactive methionine used in the experiment. By comparing the counts given by an unknown sample with the counts given by the methionine standard (after correcting each for background and self-absorption), the amount of the administered methionine represented by the unknown was calculated by a simple proportion. The per cent of administered methionine, methyl group, or C^{14} (in this instance all three are identical) that had been converted to or was contained in the unknown was then calculated.

Organ and Tissue Analysis—At 52 hours the rat was anesthetized with sodium amytal, exsanguinated, skinned, and dissected. The intestinal tract was washed out with water. After weighing the organs, samples ranging in weight from 25 to 150 mg. were removed for combustion. The weights of the more diffuse tissues were calculated from the figures given by Donaldson (10). The weights of tissues and organs together with their C^{14} content (in terms of the 200 mg. dose of radioactive methionine) are given in Table II. The sum of these weights, 143 gm., compares favorably with the weight of the animal at the time of sacrifice, 141 gm. The concentration of C^{14}, in terms of administered methionine, in the fresh tissues and organs is illustrated in Fig. 2.

[5] When necessary, non-radioactive Na_2CO_3 was added to the NaOH solution to provide the desired amount of carbonate for precipitation with $BaCl_2$.

Chemical Fractionation—The procedure followed in the solvent fractionation of the rat (less kidneys, blood, and samples taken for combustion) is illustrated in Diagram 1. Several extractions were made with each solvent. The concentration of C^{14} (in terms of administered methionine) found in each of these fractions is shown in Table III.

In addition to the fractionation shown in Diagram 1, the ether-soluble fraction was saponified in $Ba(OH)_2$ solution and acidified. The lipides

TABLE II

Distribution of Labeled Methyl Carbon Administered As Methionine (200 Mg.) in Organs and Tissues at 52 Hours

Tissue	Wet weight	Labeled carbon*
	gm.	*mg.*
Muscle.......	63.5†	27.9
Liver..	7.2	23.7
Integument.................................	24.6†	12.6
Small intestine.............................	4.3	5.8
Blood........... ....†	10.0†	5 5
Bone..	10.2†	6.35
Kidneys.....................................	1.3	5.2
Large intestine.............................	3.5	3.2
Testes......................................	3.3	3.1
Lungs........	3.7	2.8
Depot fat.....	8.5†	1.2
Spleen........	0.9	0.95
Pancreas....................................	0.8	0.76
Brain.......................................	1.5†	0.5
Adrenals....................................	0.025	0.06
Intestinal contents.........................		1.4
Total.....................................	143.2	101.02

* The labeled carbon is expressed as mg. of administered methionine.
† Calculated from the data given by Donaldson (10).

were then removed from the water-soluble components (choline, glycerol, etc.) by ether extraction. The lipide fraction was resaponified in a methanol solution of KOH and separated into fatty acids and non-saponifiable material. The fatty acids were only slightly radioactive. The ethereal solution of the non-saponifiable matter was washed twice with 0.1 N NaOH and seven times with water. It was then subdivided into sterol, wax, and oil fractions by means of fractional crystallization from methanol (11). The cholesterol fraction after two crystallizations from methanol yielded a white crystalline material composed of notched monoclinic

plates.   The solid fraction (wax) was pale yellow and the oil fraction was red.   Each of these fractions possessed approximately the same low degree of radioactivity.

Isolation of Choline and Creatine—Choline was isolated from both the water- and ether-soluble fractions as choline chloroplatinate.   Creatine was isolated from the water-soluble fractions as creatinine potassium
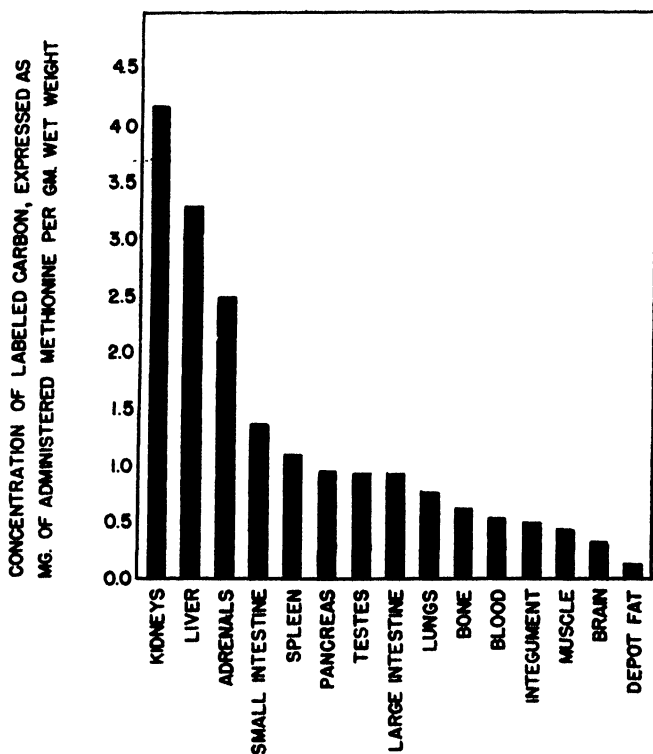
Fig. 2. Concentration of the labeled methyl carbon administered as methionine in the organs and tissues at 52 hours.

picrate (3).   The platinum content of each of the choline derivatives was 31.2 per cent as compared with the theoretical value of 31.7 per cent. The creatinine potassium picrate was pure, according to the Jaffe reaction.

The creatine content of the water-soluble and trichloroacetic acid-soluble fractions (Diagram 1) as determined by the Jaffe reaction was 223 mg. and 72 mg., respectively.   The choline chloroplatinate isolated from the water- and ether-soluble fractions amounted to 36 mg. and 154

DIAGRAM 1

Procedure for solvent extraction

Rat frozen in dry ice, ground, and
extracted with absolute alcohol,
hot 95% alcohol, warm alcohol-
ether, and ether

Combined extracts

Alcohol and ether removed *in
vacuo*, extract partitioned be-
tween water and ether

Residue

Extracted with 6% trichloroacetic
acid

Water-soluble

Dried *in vacuo*

Ether-soluble

Ether removed *in vacuo*

Residue

Extracted with acetone and
ether; ether fraction dis-
carded

Trichloroacetic acid extract

Extracted with ether to remove
TCA; ether fraction discarded

Acetone-soluble

Acetone removed *in vacuo*; resi-
due washed with ether and
dried

Crude protein

Residue dried and ground in
mill

Trichloroacetic acid-soluble

Residual solvents removed *in
vacuo*; extracted with ether;
dried *in vacuo*

mg., respectively. These values were translated into the amount of water-soluble and fat-soluble choline contained in the whole animal on the basis of the choline content of the rat as given by Jacobi and Baumann (12).

The radioactivity of the choline and creatine samples was determined following their combustion. On the basis of our past experience it was assumed that all of the C^{14} was in the methyl groups. The C^{14} present in these compounds was expressed as mg. of administered methionine (Table III).

TABLE III

Distribution of Labeled Methyl Carbon Administered As Methionine in Chemical Fractions of Body* at 52 Hours

Fraction	Dry weight	Labeled carbon†		
		Amount	Portion present as choline	Portion present as creatine
	gm.	*mg.*	*mg.*	*mg*
Water-soluble	2.0	13.51	6.1	4.4
Ether-soluble	10.9	20.48	16.8	
Trichloroacetic acid-soluble...	17.4	8.16		1.0
Acetone-soluble	1.5	3.90		
Crude protein	18.0	30.60		
Total	49.8	76.65	22.9	5.4

* The kidneys and approximately one-half of the blood were not included in the fractionation.

† The labeled carbon is expressed as mg. of administered methionine.

SUMMARY

The results of an experiment are reported in which 200 mg. of methionine labeled with C^{14} in the methyl group were given orally to a rat that was then fasted for 52 hours.

The ability of the animal organism to oxidize the methyl group (administered as methionine) to carbon dioxide and water at a rapid rate was demonstrated by the appearance of one-quarter of the administered methyl carbon in the expired carbon dioxide during the 1st day. Thereafter the rate of oxidation of the methyl group fell to a low but steady value.

At the same time the administered methyl group participated extensively in transmethylation reactions, as was shown by its appearance in the methyl groups of creatine plus choline in an amount equal to the quantity present in the body protein itself. Moreover, the specific activity of both the water-soluble and fat-soluble choline fractions exceeded the estimated specific activity of the protein-bound methionine. Accompanying these

chemical translocations of the administered methyl group was its widespread distribution throughout the body, as reflected by the presence of C^{14} in all of the major organs and tissues. However, this distribution was most uneven, by far the highest concentrations of C^{14} being found in the kidneys, liver, and adrenals.

These findings suggest that the chemical and anatomical distribution of the administered methyl group contributed to the fall in the initial high rate of oxidation and to the establishment of the steady rate of oxidation of this radical, and, conversely, that the establishment of this steady rate of oxidation marked the end of the major chemical and morphological translocations that brought the methyl group into equilibrium with the labile methyl pools (and their oxidation products) already present in the body.

The total recovery of the administered methyl carbon was 94 per cent when calculated from the chemical analyses of the animal plus the C^{14} eliminated in the respiratory CO_2, urine, and feces. Similar calculations on the basis of tissue and organ analyses gave a total recovery of 101 per cent.

The authors wish to acknowledge the technical assistance of Mrs. Marion H. Wilson.

BIBLIOGRAPHY

1. du Vigneaud, V., *Harvey Lectures*, **38**, 39 (1942–43).
2. Schenck, J. R., Simmonds, S., Cohn, M., Stevens, C. M., and du Vigneaud, V., *J. Biol. Chem.*, **149**, 355 (1943).
3. du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S., *J. Biol. Chem.*, **140**, 625 (1941).
4. Keller, E. B., Rachele, J. R., and du Vigneaud, V., *J. Biol. Chem.*, **177**, 733 (1949).
5. Melville, D. B., Rachele, J. R., and Keller, E. B., *J. Biol. Chem.*, **169**, 419 (1947).
6. Mackenzie, C. G., Chandler, J. P., Keller, E. B., Rachele, J. R., Cross, N., Melville, D. B., and du Vigneaud, V., *J. Biol. Chem.*, **169**, 757 (1947).
7. Bloch, K., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **138**, 155 (1941).
8. Block, R. J., and Bolling, D., The amino acid composition of proteins and foods, Springfield (1945).
9. Henriques, F. C., Jr., Kistiakowsky, G. B., Margnetti, C., and Schneider, W. G., *Ind. and Eng. Chem., Anal. Ed.*, **18**, 349 (1946).
10. Donaldson, H. H., The rat, Memoirs of The Wistar Institute of Anatomy and Biology, Philadelphia, 2nd edition (1924).
11. Mackenzie, C. G., Mackenzie, J. B., and McCollum, E. V., *Pub. Health Rep., U. S. P. H. S.*, **53**, 1779 (1938).
12. Jacobi, H. P., and Baumann, C. A., *J. Biol. Chem.*, **142**, 65 (1942).

THE ACTIVITY OF SUCCINATE OXIDASE IN RELATION TO PHOSPHATE AND PHOSPHORUS COMPOUNDS*

By ERIC G. BALL AND OCTAVIA COOPER

(From the Department of Biological Chemistry, Harvard Medical School, Boston)

The usual procedure for the preparation of succinate oxidase involves the extraction of washed heart muscle with a mildly alkaline phosphate solution. A turbid dispersion is thus obtained which is resistant to precipitation by a low centrifugal force and which contains at least cytochrome oxidase, cytochromes *a*, *b*, *c*, and a flavoprotein. This enzyme preparation, besides catalyzing the oxidation of succinate and *p*-phenylenediamine, can also be shown to be capable of gearing a pyridine nucleotide enzyme system to oxygen. It thus contains a major portion of the enzymes involved in the transfer of electrons and hydrogen ions from substrates to oxygen. In order to study the composition of this integrated enzyme complex and the possible formation of high energy phosphate compounds during its functioning, it seemed worth while to attempt its preparation by a procedure which did not involve the use of phosphate solutions. We describe here such a procedure along with some interesting properties of the resulting enzyme preparation.

EXPERIMENTAL

Preparation of Enzyme—All steps to be described, including centrifugation, are carried out at about 4° and completed within 1 day. The centrifugal speeds which are given apply to the size 2 International centrifuge with a four-place head carrying 250 cc. cups. Glass-redistilled water was employed throughout. We have avoided the use of pig heart as a starting material, since we have experienced trouble with excessive amounts of copper in such hearts, presumably due to the use of massive doses of copper sulfate as a vermicide just prior to the slaughter of these animals.

Step A; KCl Extract—50 gm. of fresh beef heart are put through a meat grinder twice, then mixed with 200 cc. of ice-cold water, and blended in a Waring blendor for 2 minutes. This homogenate is centrifuged for 10 minutes at 1900 R.P.M. and the clear red supernatant discarded. The residue is mixed and blended for 30 seconds with 200 cc. of an ice-cold extract of heart muscle. (This extract is prepared by boiling 50 gm. of

twice ground heart muscle in 200 cc. of water for 10 minutes and filtering.) If water is used for washing at this stage, a turbid supernatant is obtained with a consequent loss of enzyme. The resulting suspension is centrifuged for 10 minutes at 1900 R.P.M. and the supernatant again discarded. The residue is then mixed and blended for 30 seconds with ice-cold water, centrifuged 10 minutes at 1900 R.P.M., and the clear, slightly colored supernatant discarded. The residue is now blended with 200 cc. of cold 1.0 M KCl for 30 seconds. This suspension is centrifuged for 30 minutes at 2750 R.P.M. and the cloudy supernatant, which contains the enzyme, is carefully drawn off by suction. The pH of the supernatant is 5.8, its average dry weight 9 mg. per cc., and the average Q_{O_2} (c.mm. of O_2 per hour per mg. of dry weight) with succinate as substrate is 179; yield 40 to 50 per cent.

Step B; Dilution—The KCl extract is diluted with 4 volumes of cold water, added slowly, while stirring. After standing for 5 minutes, the white precipitate is removed by centrifugation (10 minutes at 2200 R.P.M.), and the supernatant which contains the enzyme is drawn off by suction. Its average dry weight is 1.0 mg. per cc. and the average Q_{O_2} with succinate as substrate is 290; yield 75 per cent.

Step C; Ammonium Sulfate Precipitation—To each 100 cc. of diluted KCl supernatant are added, while stirring slowly, 70 cc. of an ammonium sulfate solution saturated at room temperature, containing 5 cc. per liter of concentrated ammonium hydroxide. This suspension, after standing 5 minutes, is centrifuged for 15 minutes at 2600 R.P.M. and the supernatant drawn off and discarded. Enough ice-cold water is added to the precipitate to give a final volume one-tenth that of the starting diluted KCl supernatant. The pH of the preparation, which is about 7.45, is adjusted to 6.5 or 6.6 by the addition, with careful stirring, of 0.1 N HCl; 0.4 cc. per 10 cc. of enzyme preparation is usually required. Its average dry weight is 5.9 mg. per cc. and the average Q_{O_2} with succinate as substrate is 407; yield 90 per cent.

The preparation at this stage is opalescent to turbid in appearance. When viewed with a hand spectroscope, it shows no oxyhemoglobin or oxymyoglobin bands. On addition of $Na_2S_2O_4$, the bands of reduced cytochrome a, b, and c become clearly visible and are about equal in intensity. Adjustment of the pH of the preparation at this stage to 6.5 minimizes the loss of activity that occurs on storage. Even so, the preparation loses activity rapidly when stored at 4° for reasons that will become apparent.

Chemical Determinations—Measurements of oxygen consumption were made by the conventional Warburg manometric procedure at 37.1°. The gas phase was air except when bicarbonate buffers were employed; then a 5 per cent CO_2-95 per cent air mixture was used. Sodium succinate

and p-phenylenediamine were employed as substrates at final concentrations of 0.013 M and 0.015 M, respectively. Cytochrome c was added to all flasks to yield a final concentration of 10^{-5} M.

Iron determinations were made by a modification of the dipyridyl method described by Keilin and Hartree (9). To insure proper pH for the development of the color, 1 M acetate buffer, pH 4.6, was added.

Phosphate determinations were made by the method of Fiske and Subbarow (5) with the aid of a Klett photoelectric colorimeter. Fractionation of the enzyme preparation into different phosphorus fractions was carried out by the method of Schneider (14).

The determination of the dry weight of enzyme preparations was performed on samples submitted to exhaustive dialysis or to precipitation by the addition of an equal volume of 95 per cent ethyl alcohol with three washings of the precipitate with 50 per cent alcohol. Enzyme samples which were run by both techniques gave identical dry weight values.

Flavin-adenine dinucleotide was determined by means of a coenzyme-free D-amino acid oxidase system in the manner first described by Warburg and Christian (18) with use of a pure sample of flavin-adenine dinucleotide as a standard. The analyses were performed on extracts made by heating the enzyme preparations at 90° for 10 minutes, cooling immediately, and centrifuging.

Results

The values reported for Q_{O_2} in the description of the method for the preparation of the enzyme were determined in 0.1 M phosphate buffer, pH 7.4. The activity of the enzyme is, however, markedly affected by the type of buffer system employed, especially at the final step of the preparation. In Table I data are presented which illustrate this point. The enzyme shows little or no activity in the oxidation of succinate when bicarbonate or glycerophosphate buffers are employed, while its activity in glycylglycine is about 50 per cent of that observed in a phosphate buffer. When the cytochrome oxidase activity of the same enzyme preparation is measured with p-phenylenediamine as substrate, there is a high and equal activity observed in all four buffers.

Data of this sort at first suggested that inorganic phosphate was in some way involved in the oxidation of succinate. Indeed, the addition of inorganic phosphate to bicarbonate buffer did improve the activity of the enzyme system. However, as can be seen from the data presented in Table II, relatively large quantities of phosphate were needed to give an appreciable increase in activity. It seemed unlikely, therefore, that this action of inorganic phosphate could be ascribed to a need for it in the oxidative process. Consonant with this interpretation is the greater activity

TABLE I

Activity of Succinate Oxidase in Different Buffer Systems

Buffer system	pH	Oxygen consumption
		c.mm. per hr.
0.1 M Na_2HPO_4-KH_2PO_4	7.26	101
0.1 " glycylglycine	7.28	53
0.1 " glycerophosphate	7.32	13
0.028 M $NaHCO_3$-5% CO_2, 0.13 M KCl.	7.31	6

Each manometric flask contained 2.0 cc. of buffer solution; 0.1 cc. of cytochrome c, 3×10^{-4} M; 0.2 cc. of sodium succinate, 0.2 M; 0.1 cc. of enzyme from Step C; and water to make a total volume of 3.0 cc. Temperature 37.1°. The pH values were determined by means of a glass electrode on the contents of the flasks at the end of the run. The succinate was tipped from the side arm after 20 minutes temperature equilibration.

TABLE II

Effect of Addition of Phosphate Ions, Glycine, and Aluminum and Calcium Ions on Succinate Oxidase Activity in Various Buffers

Experiment No.	Buffer system	Addition	Oxygen consumption
			c.mm. per hr.
I	Bicarbonate, 0.028 M		0
	" 0.028 "	0.01 cc. 0.1 M PO_4, pH 7.3	5
	" 0.028 "	0.1 " 0.1 " " " 7.3	11
	" 0.028 "	0.5 " 0.1 " " " 7.3	57
	Phosphate, 0.1 M		85
II	Bicarbonate, 0.028 M		0
	" 0.028 "	0.5 cc. 1.2 M glycine	120
	Phosphate, 0.1 M		107
	" 0.1 "	0.5 cc. 1.2 M glycine	196
	Glycerophosphate, 0.1 M		6
	" 0.1 "	0.5 cc. 1.2 M glycine	184
III	Bicarbonate, 0.028 M		0
	" 0.028 "	0.3 cc. 4×10^{-3} M $AlCl_3$	52
	" 0.028 "	{ 0.3 " 4×10^{-3} " " { 0.3 " 4×10^{-3} " $CaCl_2$	30
	Phosphate, 0.1 M		114
	" 0.1 "	{ 0.3 cc. 4×10^{-3} M $AlCl_3$ { 0.3 " 4×10^{-3} " $CaCl_2$	168

Experimental details similar to those described for the data in Table I except that the given addition replaced an equal volume of water. pH values in all cases 7.4 ± 0.1. A different enzyme preparation was employed in each of the three sets of experiments, so that they are not directly comparable.

observed in glycylglycine buffer than in glycerophosphate, since inorganic phosphate is a more likely impurity in the latter than in the former.

The behavior of glycylglycine buffer suggested a trial of the effects of amino acids upon the process. The addition of glycine to a bicarbonate or glycerophosphate buffer causes a pronounced increase in the activity of the enzyme, as can be seen from the data given in Table II. The response obtained increases as the glycine concentration increases and begins to level off at the concentration given in Table II. The addition of glycine to phosphate buffer also markedly improves the activity of the enzyme, as the data in Table II demonstrate. No oxygen consumption is observed with glycine in the absence of succinate.

The observations of Bodansky (3) on the inhibition of phosphatases by various amino acids came to our attention at this time. We were led, therefore, to test the action of alanine and histidine on succinate oxidase activity. Alanine is somewhat less effective than glycine, but histidine is as effective as glycine at one-fiftieth the concentration. These results resemble those reported by Bodansky for the effectiveness of these three amino acids in the inhibition of phosphatases. A working hypothesis was therefore formulated which postulated the presence of a phosphatase in our enzyme preparation. This phosphatase, if uninhibited, is pictured as acting upon some phosphorylated compound essential to the activity of succinate oxidase. Thus the lack of activity in bicarbonate buffer would be explained by a destruction of this component of the enzyme system during the 20 minute period of temperature equilibration that precedes a measurement. The addition of inorganic phosphate or amino acid prevents this destruction by inhibiting the phosphatase.

Proof for this hypothesis was therefore sought. The lack of activity in bicarbonate buffer could readily be shown to be due to the destruction of some component of the enzyme system during the equilibration period. For example, addition of inorganic phosphate or amino acid to the enzyme-bicarbonate buffer mixture after, instead of before, the equilibration period was ineffective. Also, addition of the enzyme from the side arm of the flask into the buffer substrate mixture after temperature equilibration produced some activity which, however, rapidly decreased as the experiment progressed. Furthermore, shortening of the equilibration period favored the retention of activity in bicarbonate buffer. There thus seemed little doubt that a destruction of some component of the succinate oxidase complex explained the results obtained with bicarbonate buffer. Similar results were also obtained with glycerophosphate buffer.

Interestingly enough, aluminum ions also protect the enzyme from destruction in bicarbonate buffer. Horecker, Stotz, and Hogness (8) were the first to show that aluminum ions enhanced the activity of succinate

oxidase.   Potter and Schneider (13) confirmed this finding.   Both of these groups of investigators interpreted their findings as indicating a rôle for aluminum in the oxidative mechanism.   It seems possible, in the light of our results, that aluminum acts by protecting the succinate oxidase system from destruction rather than as a stimulant *per se*.   Thus its action would be not unlike that of calcium on this system.   Swingle *et al.* (16) present evidence that calcium increases the succinate oxidase activity of tissue homogenates by activating the cozymase nucleotidase present in the homogenates.   Cozymase is thus more rapidly destroyed, and the formation of oxalacetate, which is strongly inhibitory to the succinoxidase system, is prevented.

Calcium shows no stimulatory effect on our enzyme preparation and, indeed, tends to counteract the beneficial effects of aluminum.   We may interpret this to mean that oxalacetate-forming enzymes are already absent from our system and that calcium therefore now acts chiefly to activate the phosphatase which aluminum tends to inhibit.   Magnesium and manganese ions behave in a similar fashion.

We have sought further to test the correctness of the hypothesis that a phosphatase is responsible for the destruction of succinate oxidase in bicarbonate buffer by the use of fluoride, which is an accepted inhibitor of phosphatases.   A direct manometric test of the action of fluoride is, however, not possible, since fluoride also inhibits the succinic oxidase system. We have, therefore, proceeded in the following manner.   The succinate oxidase system is not inhibited by fluoride if its final concentration in the manometer flasks does not exceed 0.002 M.   Now, since only 0.1 cc. of the final enzyme preparation is needed for a test of activity in a final volume of 3.0 cc., it is possible to make the enzyme preparation itself 0.05 M in NaF without affecting its activity in the ultimate test system.   The effect of fluoride on the stability of the enzyme preparation during storage can thus be observed.

In Fig. 1, the data from an experiment of this type are presented.   Four portions of the enzyme as precipitated in Step C were taken up in four different solutions which had the final composition indicated.   The activity of each was determined in phosphate buffer as described previously and the preparations were then stored at 4°.   On subsequent days, as indicated in Fig. 1, their activity was again determined.   The results show that fluoride at a final concentration of 0.05 M has a pronounced effect on the keeping qualities of the enzyme; a combination of 0.1 M PO_4, pH 6.6, and 0.05 M NaF, however, provides optimum conditions.   These findings thus tend to confirm the hypothesis that a phosphatase is exerting a destructive effect upon the succinate oxidase complex.   They also, however, furnish a practical means of preserving the succinate oxidase system during storage.

We are now routinely taking up our final enzyme preparation in 0.1 M phosphate buffer, pH 7.0, containing 0.05 M NaF for further studies on its composition.
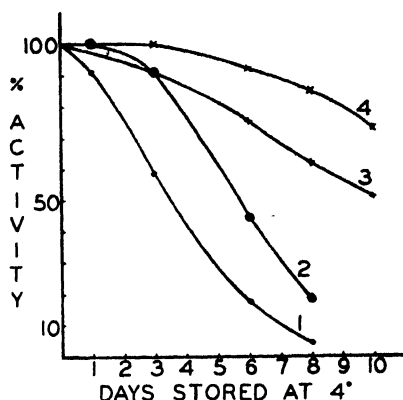
FIG. 1. Activity of succinate oxidase preparation from Step C after storage at 4° in various media. Curve 1, in H_2O, pH 6.47; Curve 2, in 0.1 M phosphate, pH 6.58; Curve 3, in 0.05 M NaF, pH 6.25; Curve 4, in 0.1 M phosphate and 0.05 M NaF, pH 6.57.

TABLE III

Analytical Data on Various Preparations of Enzyme, Step C

	Preparation No.								Average
	1	2	3	4	5	6	7	8	
Dry weight, *mg. per cc.* .. .	4.5	7.2	6.2	6.6	4.7	6.7	5.7	5.8	5.9
Q_{O_2}	552	314	455	309	343	397	447	435	407
Fe content, *% dry weight*. ..	0.11	0.059							0.084
Flavin-adenine dinucleotide, *% dry weight*.		0.024		0.027					0.0255
Total P, *% dry weight*... ..		1.33	1.64	1.50	1.47	1.51	1.70	1.62	1.54
Total acid-soluble P, *% total P*		0	0.9	3.2	2.2	9.3	3.1	6.2	3.6
Nucleic acid P, *% total P* ...					28.7	27.2	30.5	29.5	29.0
Phospholipide P, *% total P*...						51.9	76.3	73.5	67.2

Q_{O_2} values determined in 0.1 M PO_4 buffer, pH 7.4.

In view of the findings described above, which suggested that a phosphorylated compound was important to the functioning of succinate oxidase, we have analyzed our final preparations for their phosphorus content. The results are given in Table III. The total phosphorus content is surprisingly high and averages about 1.5 per cent of the dry weight.

Approximately two-thirds of this phosphorus is present in a form soluble in lipide solvents, and most of the remainder occurs as nucleic acid phosphorus. A content of 1 per cent phospholipide phosphorus would indicate that a large percentage of our enzyme preparation is composed of lipide material. This is indeed the case. Extraction of dried salt-free enzyme preparations with a hot alcohol-ether mixture has uniformly yielded 37 per cent of the dry weight as material soluble in this solvent mixture. On the basis of the nucleic acid phosphorus values, another 5 per cent of the dry weight may be reckoned as nucleic acid. Thus, the total protein content of our enzyme preparation is of the order of 58 per cent.

For orientation purposes, the iron and flavin-adenine dinucleotide content of two of our preparations has been determined and the data are given in Table III. The correlation between the iron values and the Q_{O_2} values is perhaps fortuitous. Though our preparations are spectroscopically free of hemoglobin and myoglobin, we cannot be certain that all of the iron found represents cytochrome-bound iron. However, reckoned on the basis of the protein content of our preparation, the average iron content approaches 0.15 per cent of the protein dry weight, or about half that present in most crystalline iron porphyrin proteins.

We have presented evidence which suggests that the rapid destruction of succinate oxidase that occurs in bicarbonate buffer is due to the action of a phosphatase. If this is true, we might expect some change to occur in the phosphorus partition during the loss of activity that occurs during enzyme aging. The following experiment indicates that this is the case. A preparation of the enzyme was carried through Step C, except that the pH was left at 7.36 to enhance the rate of enzyme destruction in storage. A drop of toluene was added and the preparation incubated at 38°. Samples were removed at the start and at various time intervals thereafter for phosphorus analyses and succinate oxidase determinations. Initial values for inorganic P and total acid-soluble P were 0.9 and 3.2 γ per cc., respectively. After $6\frac{1}{2}$ hours of incubation, the corresponding phosphorus values were 1.3 and 6.7 γ per cc. and the succinate oxidase activity was 59 per cent of its initial value. At the end of 24 hours of incubation, all succinate oxidase activity had disappeared, and the inorganic P was then 4.0 γ per cc. and total acid-soluble P 11.2 γ per cc. The increase in total soluble P during the 24 hours thus amounts to 8.0 γ per cc. or less than 10 per cent of the total P of 99 γ per cc. The flavin-adenine dinucleotide content of the preparation was also followed during the incubation period. Values of 1.75, 1.23, and 1.00 γ per cc. were found at zero, $6\frac{1}{2}$, and 24 hours. In other experiments of this nature, the flavin-adenine dinucleotide content has dropped to zero in 24 hours. Thus there occurs both a conversion of a small portion of the non-acid-soluble P into acid-soluble and inorganic

P and a destruction of flavin-adenine dinucleotide during the loss of succinate oxidase activity.

Findings such as these naturally suggest that it might be possible to prevent destruction of succinate oxidase activity or even to restore activity in our preparation by the addition of the proper phosphorylated compound. The addition of adenosine triphosphate (ATP), diphosphopyridine nucleotide, thiamine pyrophosphate, adenosine-3-phosphate, adenosine-5-phosphate, flavin-adenine dinucleotide, yeast nucleic acid, α-tocopherol phosphate, or heated heart muscle extract did not restore activity to a preparation that had undergone inactivation in bicarbonate buffer during a manometric test. The addition of an extract of the water-soluble protein of heart muscle along with ATP was also without restorative properties. The addition of ATP to the bicarbonate buffer before the enzyme, however, did afford some protection to the enzyme. Its action in this respect was not outstanding and suggested that it acted by competing for the phosphatase rather than as a component of the succinate oxidase system.

DISCUSSION

The association of considerable lipide material with the various enzymes known to compose the succinate oxidase complex raises some interesting points. In the first place, it would appear that the combination between protein and lipide material is a relatively tight one. Not only does the union of the two components survive the fractionation procedure described here, but it also resists rupture by high speed centrifugation. Preparations of the enzyme (Step C), when submitted to centrifugation at 18,000 R.P.M. (25,000 $\times$ g) for 1 hour at 0°, form translucent brown pellets in the bottom of the lusteroid centrifuge tubes and leave a practically clear and colorless supernatant. The precipitate possesses nearly all the lipide material and enzyme activity of the original preparation. The differences in specific gravity of lipide and protein material are thus not sufficient to rupture their association in this complex. Furthermore, the uniformity from preparation to preparation of the total P values (chiefly lipide P) suggests that the content of lipide and protein material tends to bear a fairly constant ratio one to the other. Approximate calculations of this ratio on a molecular basis may be made. The average value found for the weight ratio of lipide to protein in the complex is 37:58. If a molecular weight of 700 is assumed for the lipide material, it is possible to calculate that with each protein molecule of molecular weight of 100,000 there will be associated 90 lipide molecules.

A rôle for the phospholipides in oxidative processes has long been postulated (cf. Sinclair (15)). Whether the phospholipides play such a rôle in the functioning of the succinate oxidase system cannot be answered at this

time. Another rôle, more passive in nature, which may be suggested for them is the holding together of a group of enzymes which interact with one another in a chain of reactions. As pointed out previously (2), we are dealing in the succinate oxidase complex with enzymatic reactions in which one enzyme is the substrate for the other. The efficiency to be achieved in such a system by the spatial fixation of the participating enzymes close to one another is obvious. Thus the phospholipides might be looked upon as a cement substance to hold in close association groups of enzymes that perform together in metabolic cycles. In this connection, the location in the mitochondria of the succinate oxidase system (7), fatty acid oxidase (11), tricarboxylic acid cycle enzymes (11), along with the high phospholipide content (12) of these granules, is suggestive.

The postulate that this lipide or phospholipide material is responsible for binding together the various oxidative enzymes found in this complex finds support in the action of desoxycholate upon this complex. Keilin and Hartree (10) and Wainio et al. (17) have used desoxycholate solutions either to clarify or to solubilize certain enzyme components of the succinate oxidase system. We have found that the addition of desoxycholate to our enzyme preparation to give a final concentration of 2 per cent changes its properties pronouncedly. High speed centrifugation now separates only a small fraction of the original material and the supernatant is crystal-clear and light brown in color. Its absorption spectrum can be quantitatively determined and clearly shows the presence of cytochromes a and b upon addition of $Na_2S_2O_4$ to the solution. We shall not discuss the enzyme properties of this preparation at this time. Let it suffice to say that all the evidence points to a dispersal and solubilization of the various components. In view of the well known interaction of desoxycholate and lipides, it does not seem unreasonable to ascribe these pronounced changes in physical properties of the enzyme preparation to a similar type of interaction of desoxycholate with the lipide molecules of the enzyme complex.

Another aspect of the association of considerable lipide material with the succinate oxidase complex that deserves mention is the inhibition of its activity by 2-hydroxy-3-alkylnaphthoquinones (2, 6). The effectiveness of these compounds as inhibitors is related to the length and type of the alkyl groups they contain. In general it can be said that the more lipide-soluble the substituted group the more effective the compound becomes as an inhibitor. Is it possible that these naphthoquinones are so effective as inhibitors by reason of their affinity for the lipides of the succinate oxidase complex? Not without interest in this connection are calculations which show that the amount of succinate oxidase (0.7 mg. per 3.0 cc.) employed in the final enzyme test system results in a concentration of lipides (molecular weight of 700 assumed) of about 10^{-4} M in this system. Some of

the naphthoquinones are effective inhibitors in such a test system at concentrations as low as 10^{-5} to 10^{-6} M.

The data presented here suggest the participation of a phosphorylated compound in the chain of reactions that occur when succinate is oxidized by molecular oxygen. This phosphorus compound would appear to function somewhere between succinate and cytochrome c, since the oxidation of p-phenylenediamine does not appear to be dependent on its presence. This phosphorylated compound also appears to be necessary for the reaction of succinate with methylene blue. The chemical nature and exact rôle of this phosphorylated compound is unknown. The only known phosphorus-containing coenzyme present in the enzyme complex is flavin-adenine dinucleotide. The observed concomitant, though not always complete, destruction of this coenzyme and loss of succinate oxidase activity suggest that a flavoprotein might be involved. Yet the addition of flavin-adenine dinucleotide does not restore activity once it is lost. Such a result might be expected, however, if flavin-adenine dinucleotide was split by a phosphodiesterase without the release of the resulting mononucleotides from the protein moiety.

Axelrod *et al.* (1) have suggested that a flavoprotein is involved in the succinate oxidase system. Indeed, for all we know, succinate dehydrogenase may be a flavoprotein, since the exact nature of the enzyme is still unknown. It also is possible that a flavoprotein is necessary to link succinate dehydrogenase to cytochrome c, since evidence is accumulating that some linkage is necessary (*cf.* (2)). Experiments now in progress may help to elucidate the nature of the phosphorylated compound essential to the functioning of the succinate oxidase system.

The observations made here that amino acids can protect an enzyme from destruction during *in vitro* studies may not be without a wider application. We should like to suggest that the observations of Bodansky (3), that amino acids are effective inhibitors of phosphatases, may provide us with a physiological reagent for stabilizing tissue slices or homogenates against the loss of important phosphorylated compounds and so permit *in vitro* metabolic studies not yet achieved. Indeed, in view of the findings of Christensen *et al.* (4) on the concentrations of amino acids within tissues, we may even wonder whether they may not play some regulatory rôle *in vivo*.

SUMMARY

1. A preparation of succinate oxidase has been obtained from heart muscle by a method which does not involve the use of phosphate buffers. Such preparations show little or no activity as measured manometrically in bicarbonate or glycerophosphate buffers, moderate activity in glycyl-glycine buffers, and high activity in phosphate buffers.

2. It is shown that lack of activity in bicarbonate buffer is due to the destructive action of a phosphatase also present in the preparation. This destructive action can be retarded by phosphate or aluminum ions, amino acids, or fluoride. The nature of the essential phosphorylated compound destroyed is not known, but there is a possibility that flavin-adenine dinucleotide is involved.

3. Analysis of the enzyme preparation shows that 37 per cent of its dry weight is soluble in lipide solvents and that 1 per cent of the dry weight is lipide phosphorus. The possible significance of this finding in relation (1) to the action of desoxycholate and naphthoquinones on the enzyme system, and (2) to the binding together of the various enzymes in the complex is discussed.

BIBLIOGRAPHY

1. Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 297 (1942).
2. Ball, E. G., Anfinsen, C. B., and Cooper, O., *J. Biol. Chem.*, **168**, 257 (1947).
3. Bodansky, O., *J. Biol. Chem.*, **174**, 465 (1948).
4. Christensen, H. N., Streicher, J. A., and Elbinger, R. L., *J. Biol. Chem.*, **172**, 515 (1948).
5. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
6. Heymann, H., and Fieser, L. F., *J. Biol. Chem.*, **176**, 1359 (1948).
7. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.*, **172**, 619 (1948).
8. Horecker, B. L., Stotz, E., and Hogness, T. R., *J. Biol. Chem.*, **128**, 251 (1939).
9. Keilin, D., and Hartree, E. F., *Biochem. J.*, **39**, 289 (1945).
10. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **127**, 167 (1939).
11. Kennedy, E. P., and Lehninger, A. L., *J. Biol. Chem.*, **172**, 847 (1948).
12. Lazarow, A., *Biol. Symposia*, **10**. 17 (1943).
13. Potter, V. R., and Schneider, W. C., *J. Biol. Chem.*, **142**, 543 (1942).
14. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
15. Sinclair, R. G., *Physiol. Rev.*, **14**, 351 (1934).
16. Swingle, K. F., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 581 (1942).
17. Wainio, W. W., Cooperstein, S. J., Kollen, S., and Eichel, B., *J. Biol. Chem.*, **173**, 145 (1948).
18. Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 150 (1938).

THE MICROBIOLOGICAL ESTIMATION OF LACTOBACILLUS LACTIS DORNER ACTIVITY WITH VITAMIN B_{12} AS A STANDARD

By MURIEL C. CASWELL, LEAH K. KODITSCHEK, and DAVID HENDLIN

(From the Research Laboratories, Merck and Company, Inc., Rahway, New Jersey)

Lactobacillus lactis Dorner has been reported to require two unidentified growth factors; one, a tomato juice component (TJ), and the other a component of liver extract, designated LLD (1, 2). Recently Shorb (3) and Rickes *et al.* (4) have shown that vitamin B_{12} was either wholly or partially responsible for the LLD activity of liver preparations. More recently Shive *et al.* (5) and Wright *et al.* (6) have demonstrated the ability of thymidine to satisfy the LLD requirement of *L. lactis* Dorner.

The extreme interest in vitamin B_{12} due to its efficacy in pernicious anemia therapy (7) has prompted us to present the method of assay used in the Merck laboratories. It is the purpose of this paper to describe in detail a microbiological assay procedure for vitamin B_{12} with *L. lactis* Dorner and to discuss some of the factors influencing the assay.

EXPERIMENTAL

Medium—The basal medium used in the assay procedure is a modification of the medium reported by Stokes *et al.* (8, 9) for the microbiological assay of amino acids. The composition of the assay medium is shown in Table I. Stock solutions of the amino acids, Salts A and B, and the vitamins were prepared as described by Stokes *et al.* (8, 9). All solutions were stored in the refrigerator under toluene except solutions of tyrosine, norleucine, and cystine. These amino acids were prepared as a mixture and stored under toluene at room temperature because of their low solubility at 5°. A stock solution containing 100 mg. per ml. of sulfuric acid- or hydrochloric acid-hydrolyzed casein was used as the source of casein hydrolysate.

The basal medium was prepared by first combining all the constituents of the medium, with the exception of sodium ethyl oxalacetate, growth factors, casein hydrolysate, and Salts A and B. After clarification by gentle heating, the mixture was adjusted to pH 7.0 with 5 N NaOH and sodium ethyl oxalacetate, growth factors, casein hydrolysate, and Salts A and B were added, and the medium adjusted to pH 6.6. The above procedure was followed in order to eliminate the possible destruction of some of the growth factors by the acidity due to constituents of the medium.

Inoculum—The stock culture of *L. lactis* Dorner variant 6a[1] was maintained in an agar stab of the following composition: Bacto-yeast extract, 1 per cent; anhydrous dextrose, 1 per cent; Bacto-agar, 1.5 per cent; tomato juice eluate,[2] 0.02 per cent; and distilled water to volume. The stab was incubated at 37° for 24 hours and then stored at 4° for further use. It

<div align="center">

TABLE I

Basal Medium (Double Strength)

</div>

DL-Leucine	100 mg.	Glucose (anhydrous)		gm
DL-Isoleucine	100 "	Fumaric acid	250	mg.
DL-Valine	100 "	Sodium ethyl oxalacetate	250	
L-Cystine	100 "	Sodium acetate, anhydrous	3	gm
DL-Methionine	100 "	Adenine		mg.
DL-Tryptophan	200 "	Guanine		
L-Tyrosine	100 "	Uracil		
DL-Phenylalanine	100 "	Pantothenic acid	100	γ
DL-Glutamic acid	100 "	Riboflavin	100	"
DL-Threonine	100 "	Thiamine·HCl	100	"
DL-Alanine	100 "	Nicotinic acid	100	"
DL-Aspartic acid	100 "	Pyridoxamine	200	"
L-Lysine	50 "	Biotin	0.2	"
L-Arginine	100 "	*p*-Aminobenzoic acid	20	"
L-Histidine	100 "	Pteroylglutamic acid*	1.0	"
DL-Serine	100 "	Salts A		
DL-Norleucine	100 "	K_2HPO_4	250	mg.
Glycine	100 "	KH_2PO_4	250	"
Casein hydrolysate†	500 "	Salts B		
		$MgSO_4 \cdot 7H_2O$	100	"
		$MnSO_4 \cdot 7H_2O$		
		NaCl		
		$FeSO_4 \cdot 7H_2O$		
		Add distilled water to	250	ml

* Pteroylglutamic acid was dissolved in 20 per cent ethanolic solution of 0.01 N NaOH.

† H_2SO_4-hydrolyzed (norit-treated) or HCl-hydrolyzed caseins were used

has been our practice to prepare fresh agar stabs weekly, since stabs older than 2 weeks occasionally failed to grow upon transfer into liquid media.

Broth medium used for preparation of assay inoculum consisted of basal medium (diluted 1:2) to which 1 unit of crystalline vitamin B_{12}[3] was added per ml. of diluted medium. A 24 hour culture prepared in 5 ml. of

[1] Culture obtained from Dr. Shorb (1).

[2] TJ eluate kindly supplied by Dr. T. R. Wood, Organic and Biochemical Research Department, Merck and Company, Inc., Rahway, New Jersey.

[3] Crystalline vitamin B_{12} was reported by Rickes *et al.* (4) to contain 11,000 units per microgram.

broth was washed twice with 5 ml. of sterile distilled water and resuspended in 10 ml. of sterile distilled water. A 1:10 dilution of the above suspension, which gave a light transmission of 90 to 95 per cent with the Evelyn colorimeter (520 mμ filter), was used as inoculum. The use of cultures older than 24 hours was found unsuitable. 48 hour cultures showed retarded response to increased dosage of vitamin B_{12} and 72 hour cultures failed to grow on transfer.

Assay Procedure—Samples to be assayed were diluted in distilled water, after adjustment to pH 7.0, to contain approximately 0.5 unit of vitamin B_{12} per ml. of solution. The sample was then added to each of five lipless, (180 $\times$ 22 mm.) test-tubes at 0.5, 1.0, 1.5, 2.0, and 2.5 ml. quantities, respectively. Distilled water was added to bring the volume to 2.5 ml., and 2.5 ml. of basal medium (see Table I) were then added to bring the total volume to 5.0 ml. The tubes were plugged with cotton and sterilized at 15 pounds pressure for only 13 minutes. Prolonged autoclaving decreased the vitamin B_{12} level required for half maximal growth. The racks of tubes can also be covered by tin-foil, instead of plugging each tube, thus facilitating rapid inoculation. Each tube was inoculated, aseptically, with 1 drop (0.05 ml.) of a *L. lactis* Dorner suspension prepared as described above. The tubes were then incubated at 37° for 40 hours. While a decrease in the incubation time did affect the maximal degree of growth permitted by a given dosage of vitamin B_{12}, incubation for longer than 40 hours had no effect on the assay values obtained.

A standard vitamin B_{12} response curve was obtained by plotting the volumes (in ml.) of 0.05 N NaOH required to neutralize the lactic acid formed by *L. lactis* from the dissimilation of glucose against graded amounts of vitamin B_{12} added to each of two replicate tubes. The amount of acid produced could also be measured potentiometrically, a response curve being obtained by plotting the dosage of vitamin B_{12} against pH. A typical response curve is shown in Fig. 1.

Some variation was encountered in the concentration of vitamin B_{12} required for half maximal growth from one assay to another, usually ranging between 0.25 and 0.5 unit per 5 ml. For this reason, occasionally not all of the five levels of the test sample fell within the range of the standard curve.

Tomato Juice Eluate—Shorb (1, 2) has reported that *L. lactis* requires a factor for growth which is found in tomato juice preparations. The use of crude preparations in assay procedures invariably causes difficulty due to their content of inhibitory and stimulatory substances. In this particular assay, further difficulty was encountered by the fact that practically all tomato juice eluates contained some LLD activity, allowing growth to occur in tubes containing no added vitamin B_{12}. It has been possible in

the medium used in this assay, in which DL-alanine is present,[4] to replace the tomato juice factor with a mixture of fumaric acid and sodium ethyl oxalacetate, thus eliminating the above difficulties. The sensitivity of *L. lactis* Dorner to vitamin B_{12} was greatly increased by this replacement. While 1.6 units of vitamin B_{12} per tube produced half maximal growth in a tomato juice-supplemented medium, only 0.38 unit per tube was required for similar response in the presence of fumaric acid and sodium ethyl oxalacetate.

Oxygen Tension—In early experiments, a total volume of 10 ml. per tube was used in the assay procedure. Under these conditions frequent occurrence of maximal growth was encountered in tubes to which no vitamin B_{12} was added. Recently, Koditschek *et al.* (10) have shown that, under
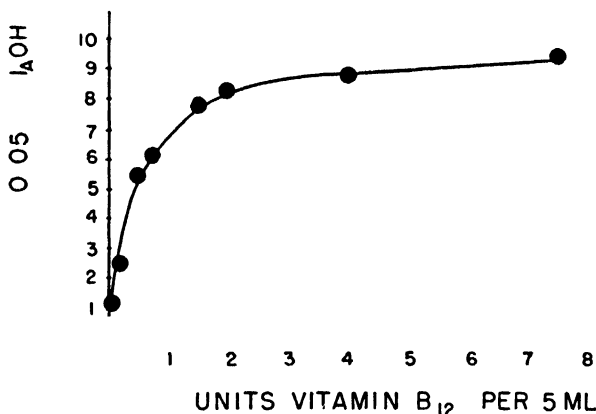
Fig. 1. *L. lactis* response to vitamin B_{12}. 1 unit is equivalent to 0.00009 γ. The points on the curve are averages of two replicate tubes.

reduced oxygen tension or low oxidation-reduction potential of the medium, added vitamin B_{12} is not required by *L. lactis* Dorner variant 6a and that increasing the volume surface area ratio decreased the vitamin B_{12} requirement. In the light of these findings, attempts were made to increase the requirement of *L. lactis* for vitamin B_{12} by increasing the O_2 tension of the medium. The medium was saturated with O_2 by shaking tubes in a reciprocal shaker before inoculation. Table II shows that shaking for 3 minutes saturated the medium with O_2. Under these conditions, the occurrence of maximal growth in tubes to which no vitamin B_{12} was added was markedly decreased but not completely eliminated. When, however, the volume of medium was decreased from 10 to 5 ml. per tube, maximal

[4] Dr. Shorb has reported by personal communication a requirement for D-alanine in a TJ-deficient medium.

growth was completely eliminated in tubes containing no added vitamin B_{12}, and greater reproducibility of assay results was obtained.

LLD-Active Substances—Recently Shive *et al.* (5) and Wright *et al.* (6) have shown that thymidine possesses LLD activity and is able to replace vitamin B_{12} for *L. lactis*. We have also observed the LLD activity of thymidine. On the basis of half maximal growth, the weight ratio of thymidine to vitamin B_{12} was found to be approximately 3000:1. This observation is in agreement with the report that 1 to 3 γ per 10 ml of thymidine is required for half maximal growth of *L. lactis* (5).

Shive *et al.* (5) have shown that ascorbic acid eliminated the requirement of *L. lactis* for added vitamin B_{12}. More recently, Koditschek *et al.* (10)

TABLE II

Effect of Shaking on O_2 Concentration of Medium

Shaking time*	Dissolved O_2†
min.	*p.p.m.*
0.0	1.0
0.03	2.1
0.25	2.8
0.50	3.9
1.0	4.8
1.5	5.3
2.0	5.9
3.0	6.4
Control‡	6.3

* Reciprocal shaker; 160 oscillations per minute.
† Determined polarographically with dropping mercury electrode.
‡ Air bubbled through the medium for 5 minutes.

have published results which substantiate these findings of Shive and have further shown that reducing substances, in general, are capable of eliminating the LLD requirement of the test organism.

We have further observed in the assay of raw materials and fermentation liquors that some of the LLD activity of these samples is due neither to reducing substance nor to vitamin B_{12}. These observations further emphasize the lack of specificity of response of *L. lactis* Dorner to crystalline vitamin B_{12}.

Statistical Analysis—An analysis of the precision of the method was made with values obtained from LLD-containing concentrates and crystalline vitamin B_{12}. These data obtained with the concentrates and crystalline material showed the same amount of variation. The precision expected for a given number of replications, *i.e.*, a given number of assay

tubes, run on more than 1 day, is presented in Fig. 2. In Fig. 2 the total number of replications is plotted against the precision range in per cent.[5] It is possible to determine from the curve the total number of tubes to be run in order to obtain a desired precision range in which one could expect repeated values on the same sample to fall 99 per cent of the time. If one desires a precision of ±20 per cent, a total of twenty tubes is required.

Day to day variation was found to be a significant factor. For this reason, values from more than 1 day should be averaged to obtain a true assay value. The number of replicates run should therefore be spread over more than 1 day.
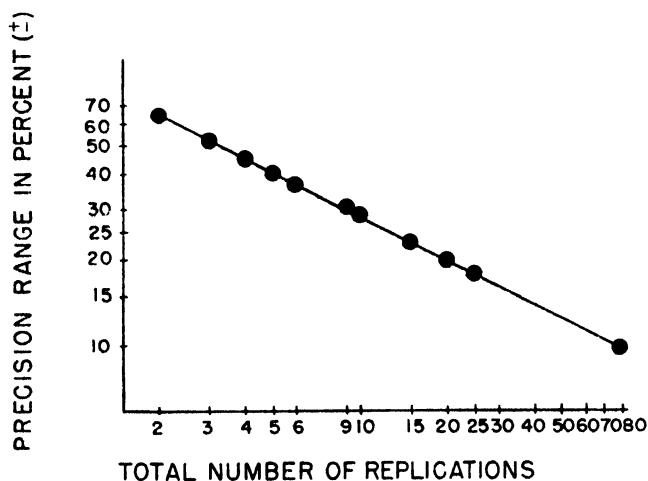
Fig 2. Precision of assay. The range within which one could expect repeated values to fall 99 per cent of the time.

SUMMARY

A microbiological method for the determination of LLD activity is described. It is based on the response of *L. lactis* Dorner to vitamin B_{12} as measured by the lactic acid formed from the dissimilation of glucose.

The specific effect of oxygen tension on the assay and means of eliminating such effects are discussed.

[5] (Precision range in per cent)^2 = $(3 \times \text{s.e.})^2$/(number of replicates).

Institute for Therapeutic Research for the statistical evaluation herein presented. The authors are indebted to Dr. H. B. Woodruff, Microbiological Section, for helpful suggestions during the course of this study.

BIBLIOGRAPHY

1. Shorb, M. S., *J. Bact.*, **53**, 669 (1947).
2. Shorb, M. S., *J. Biol. Chem.*, **169**, 455 (1947).
3. Shorb, M. S., *Science*, **107**, 397 (1948).
4 Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, **107**, 396 (1948).
5. Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, **70**, 2614 (1948).
6. Wright, L. D., Skeggs, H. R., and Huff, J W., *J. Biol. Chem.*, **175**, 475 (1948).
7. West, R., *Science*, **107**, 398 (1948).
8. Stokes, J. L., and Gunness, M., *J. Biol. Chem.*, **157**, 651 (1945).
9. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
10. Koditschek, L. K., Hendlin. D. and Woodruff, H. B., *J. Biol. Chem.*. **179**, 1093 (1949).

THE ACONITE ALKALOIDS

XXII. THE DEMETHYLATION OF DELPHININE DERIVATIVES

By WALTER A. JACOBS and YOSHIO SATO

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

Characteristic of the poisonous group of aconite alkaloids such as delphinine and aconitine is the presence in the molecule of four methoxyl groups. It was obvious from the start that demethylation would be necessary to facilitate their structural study in certain directions. In the earliest studies of this laboratory, repeated attempts were made to demethylate the bases themselves or certain of their derivatives with hydriodic acid and phosphorus or aluminum chloride, but the results proved unpromising in the cases studied. On the other hand, instances of partial demethylation (1) were encountered, mostly in the use of hydrochloric acid and in a few cases with nitric acid. Thus with α-oxodelphinine one or two methoxyl groups were replaced by Cl and a similar result was obtained with pyro-α-oxodelphinine. With each of these derivatives nitric acid was found to remove one *O*-methyl group readily but without substitution. As previously reported, such partial demethylation did not appear to occur so readily with delphinine itself or with the other derivatives tried.

In more recent work, to be described elsewhere (2), in which the Clemmensen reduction was attempted with a keto acid obtained from dihydroisopyrooxodelphonine (3), it was observed that concentration of the reaction mixture resulted in partial demethylation. This effect was then directly traced to the zinc chloride. It was soon found that a very useful reagent is a saturated zinc chloride solution in 5 per cent aqueous HCl. For general orientation the effect of this reagent at 40° was tried on octahydroisopyrooxodelphinine. The crystalline product isolated proved to be a *dimethylanhydro derivative* with the formulation $C_{29}H_{41}O_7N$ and resulted from the loss of two methyl groups and 1 mole of water. The ultraviolet absorption data obtained with this substance, as in the case of isopyrooxodelphonine and dihydroisopyrooxodelphonine as presented in Fig. 1, show essentially end-absorption. These curves are also practically superimposable on the absorption curve previously obtained (4) with α-oxodelphonine, the saponification product of α-oxodelphinine. It is apparent therefore that the double bond produced by the loss of acetic acid on pyrolysis, as well as its subsequent shift by isomerization, has not ap-

preciably affected the absorption. Attempts to hydrogenate the dimethylanhydro derivative were unsuccessful, a fact which, taken together with a similar experience with other derivatives, indicates the formation either of a resistant double bond or of an oxidic group. An oxidic group is favored by the Zerewitinoff determination, which showed the presence of only one active hydrogen due to the remaining free hydroxyl group. The loss of water appears restricted to the newly exposed hydroxyl groups and does not involve the free hydroxyl group present in octahydroisopyrooxodelphinine itself. This point will be discussed again in a succeeding
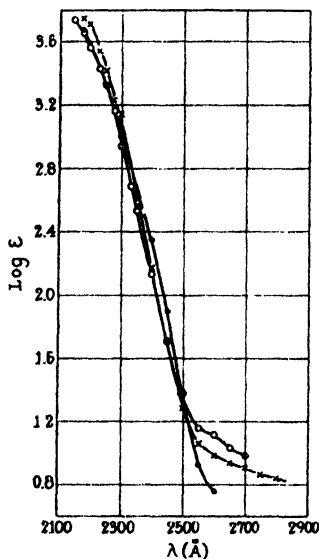
Fig. 1. Ultraviolet absorption spectra of (×) isopyrooxodelphonine, (●) dihydroisopyrooxodelphonine, and (○) the dimethylanhydro derivative in ethanol.

paper. No other demethylation products were isolated from the reaction mixture or when the reaction was tried at higher temperatures. This was in part due to the ready cleavage of the hexahydrobenzoyl group. The apparent greater resistance of the remaining methoxyl groups in octahydroisopyrooxodelphinine is suggested by the following experience with the unsaturated isopyrooxodelphinine in which a labilizing effect of the double bond is indicated.

When the reagent was tried with isopyrooxodelphinine (3) or, better, directly with pyro-α-oxodelphinine because of its initial isomerization to the former by the HCl, demethylation proceeded with unusual ease. At 40° the major reaction involved the loss of three of the O-methyl groups

and 1 mole of water. The analytical data showed the formation of the *monomethylanhydro derivative*, $C_{28}H_{31}O_7N$. The latter on hydrogenation absorbed 4 moles of H_2 with the formation of its *octahydro derivative*, $C_{28}H_{39}O_7N$. This reaction involved hydrogenation not only of the benzoyl group to hexahydrobenzoyl but of the pyro double bond, which will be discussed below. That an unsaturation, or preferably an oxidic linkage, produced by the water loss is not the point of hydrogenation is in accord with the result obtained with the demethylation product from octahydroisopyrooxodelphinine.

With the production of the monomethyl compound some complete demethylation was also noted. Further study, however, showed that this was favored at 60° with the production of *desmethylanhydroisopyrooxodelphinine*, $C_{27}H_{29}O_7N$. Because of its solubility long continued extraction with chloroform from the diluted reaction mixture was found necessary for its isolation.

A small quantity of the above intermediate monomethyl derivative was also obtained from the reaction at 60° and in addition a crystalline by-product in still smaller amount, which proved to be a chlorine-containing but completely demethylated derivative. Analysis of this *chlorodesmethylanhydro derivative* indicated a formulation $C_{27}H_{28}O_6NCl$ to be explained by the probable replacement of a hydroxyl group by chlorine.

In a previous communication (1) the production from pyro-α-oxodelphinine with HNO_3 of a substance $C_{30}H_{37}O_8N$ was described with an indicated loss of one methyl group. Because of the ready isomerizing action of the strong acid it is unquestionably an isopyro derivative. That its formation does not involve oxidation by the nitric acid used and represents an intermediate step in the demethylation series has now been shown by its complete demethylation with zinc chloride and HCl to the above desmethylanhydroisopyrooxodelphinine.

Although desmethylanhydroisopyrooxodelphinine was readily hydrogenated, the resulting octahydro derivative possessed properties which made manipulation difficult. Its study was not completed, since the experience with the saponification product proved more satisfactory, as described below. Its oxidation with chromic acid yielded a *monoketone*, $C_{27}H_{27}O_7N$, which was characterized by its *oxime*. The last to be removed and therefore the most resistant methyl group covers a secondary hydroxyl group, since the intermediate monomethylanhydro derivative, $C_{28}H_{31}O_7N$, as well as octahydroisopyrooxodelphinine, was not similarly affected by the reagent.

The desmethylanhydroisopyrooxodelphinine on saponification readily yielded *desmethylanhydroisopyrooxodelphonine*, $C_{20}H_{25}O_6N$, which proved to be soluble in water. On hydrogenation 1 mole of H_2 was absorbed with

the formation of *desmethylanhydrodihydroisopyrooxodelphonine*, $C_{20}H_{27}O_6N$. Its oxidation will be described elsewhere.

Because of the need for larger amounts of pyro-α-oxodelphinine we have studied again its preparation and purification. As presented under "Experimental," chromatography was found necessary for its satisfactory separation from by-products. The presence of a double bond in the pyro derivative was shown (3) by its hydrogenation to octahydropyro-α-oxodelphinine and by the production of an isomeric octahydroisopyro-oxodelphinine from isopyrooxodelphinine. This was supported by the fact that saponification of isopyrooxodelphinine and its octahydro derivative gave respectively isopyrooxodelphonine, $C_{24}H_{35}O_7N$, and its dihydro derivative, $C_{24}H_{37}O_7N$. This would indicate that the isomerization of pyro-α-oxodelphinine with acid is due to a shift of the double bond. More recently this conclusion has been substantiated by the failure of attempts to isomerize its hydrogenation product, octahydropyro-α-oxodelphinine, and also in the following manner.

The previously described hydrogenation product of α-oxodelphinine, hexahydro-α-oxodelphinine (5), was pyrolyzed with loss of acetic acid to *hexahydropyro-α-oxodelphinine* ($[\alpha]_D^{31} = +209°$). As expected, the latter on hydrogenation absorbed the additional mole of H_2 with the formation of the same octahydropyrooxodelphinine ($[\alpha]_D^{31} = -14.5°$), previously obtained by the one-step hydrogenation of pyro-α-oxodelphinine. The rotation found with octahydroisopyrooxodelphinine was $[\alpha]_D^{28} = -72°$.

EXPERIMENTAL

Pyro-α-oxodelphinine—For the preparation of larger quantities of this substance it was found advisable to restrict the amount of α-oxodelphinine used in each pyrolysis operation and then to combine the crude material for the isolation process.

6 gm. portions contained in 200 cc. flasks were immersed in a metal bath heated above 200° and then rapidly brought to 235–240°. The rapid, complete, and uniform melting of the substance was facilitated by the use of a spatula and the heating was continued at this temperature for a total of 12 to 15 minutes, during which complete crystallization of the melt occurred. The product from 42 gm. of starting material was dissolved in chloroform and the solution was extracted successively with dilute Na_2CO_3 and H_2O and dried. After concentration and final drying *in vacuo*, the resinous residue amounted to 33.8 gm. This material was dissolved in 400 cc. of benzene by warming and added to a column of 1 kilo of an active alumina and followed at first by 500 cc. of benzene.

Since the development of the column proceeded too slowly with 1 per cent methanol in benzene, 2 per cent was later used. After several liters

of the 2 per cent mixture had passed through, material suddenly emerged from the column. At this point the first 200 cc. yielded 2 gm. of residue and succeeding 100 cc. portions of eluent yielded in turn 1.95 gm., 2.15, 2.42, 2.62, 2.87, 3.0, 3.04, 3.5, 2.8, and 2.0 gm. Following the succeeding portions of 1.69 and 1.59 gm. of less pure material, the amount eluted dropped rapidly to 0.28, 0.12, 0.08, 0.06 gm., etc. The first eleven fractions of 28.35 gm. were combined by solution in benzene and concentrated to smaller volume and crystallization. A heavy crust of stout prisms separated which were collected with benzene. This fraction of 17.65 gm. was succeeded on concentration by a second of equally pure material of 6.54 gm. The mother liquor yielded more substance but this was contaminated by apparently unpyrolyzed α-oxodelphinine. The substance separated with or without solvent, depending upon the conditions of crystallization. In the former case, it melted at 260–262°. When crystallized from a mixture of benzene and ether, it melted gradually from 246–258°.

$$[\alpha]_D^{25} = +173° \ (c = 0.73 \text{ in } 95\% \text{ ethanol})$$
$$[\alpha]_D^{25} = +88° \ (c = 0.79 \text{ in pyridine})$$
$$C_{31}H_{39}O_3N. \text{ Calculated, C } 67.23, \text{ H } 7.10; \text{ found, C } 67.22, \text{ H } 7.15$$

Hexahydropyro-α-oxodelphinine—Hexahydro-α-oxodelphinine was obtained as previously described (5) and, when crystallized from acetone, formed nearly rectangular micro platelets which melted at 194°.

0.4 gm. of this substance was immersed in a metal bath at 200°, the temperature quickly raised to 230°, and the heating continued for 10 minutes. Although the resulting resin crystallized readily from ether, the product was contaminated with starting material and the isopyro derivative. A satisfactory separation was obtained with alumina. A solution of the material in warm benzene was concentrated to about 5 cc. and passed through 10 gm. of an active alumina. After attempted elution with about 70 cc. of benzene, this was followed with 0.5 per cent methanol in benzene, which was collected in 10 cc. portions. Of the latter, Fractions 16, 17, and 18 showed a sharp zone and gave respectively 62, 94, and 71 mg. of crystalline residue. The combined material yielded from ether 0.155 gm. of flat needles and thin leaflets, which melted under the microscope at 212–216° and again gradually partly crystallized as needles which melted at 233–242°.

$$[\alpha]_D^{25} = +209° \ (c = 0.85 \text{ in } 95\% \text{ ethanol})$$
$$C_{31}H_{45}O_3N. \text{ Calculated, C } 66.50, \text{ H } 8.11; \text{ found, C } 66.75, \text{ H } 8.20$$

Octahydropyro-α-oxodelphinine—0.1 gm. of the hexahydro derivative was hydrogenated with 50 mg. of platinum oxide catalyst in 95 per cent ethanol. Within an hour the process was practically completed, with the absorption of about 1 mole of H_2 in excess of the catalyst. The substance formed

6-sided leaflets and flat needles from ether which melted at 186–189° and showed no depression when mixed with the previously described (3) octahydro derivative obtained by hydrogenation of pyro-α-oxodelphinine.

$$[\alpha]_D^{21} = -14.5° \ (c = 0.89 \text{ in } 95\% \text{ ethanol})$$

$C_{31}H_{47}O_8N$. Calculated, C 66.26, H 8.44; found, C 66.42, H 8.55

The hydrogenation of pyro-α-oxodelphinine originally reported in acetic acid solution has since been carried out in ethanol. The resulting octahydro derivative showed $[\alpha]_D^{29} = -14°$ ($c = 0.86$ in 95 per cent ethanol). The rotation obtained with the substance prepared in acetic acid was $[\alpha]_D^{30} = -14°$ ($c = 0.86$ in 95 per cent ethanol). However, the melting point has been found to vary with the conditions of crystallization. On occasion a melting point of 199–204° after preliminary sintering has been observed.

Attempts to isomerize this substance in methanol with HCl, as in the formation of isopyrooxodelphinine (3) from pyrooxodelphinine, failed since aside from slight cleavage of hexahydrobenzoic acid it was recovered unchanged. $[\alpha]_D^{28} = -13°$.

The rotation recently found with octahydroisopyrooxodelphinine is

$$[\alpha]_D^{28} = -72° \ (c = 0.93 \text{ in } 95\% \text{ ethanol})$$

Dimethylanhydro Derivative from Octahydroisopyrooxodelphinine—0.1 gm. of octahydroisopyrooxodelphinine was treated with a solution of 4 gm. of zinc chloride in 1.4 cc. of 5 per cent HCl and the mixture was kept at 40°. Solution gradually occurred, and after 45 minutes the colorless diluted mixture was continuously extracted for several hours with chloroform. The latter yielded on concentration a resin which smelled of hexahydrobenzoic acid, but readily crystallized on solution in warm methanol-water (2:3). 29 mg. were collected in the first fraction but an additional 7 mg. were obtained in a second fraction. It formed trapezoidal micro platelets or rods from dilute methanol, which melted at 271–275°. Attempts to hydrogenate this substance were unsuccessful.

$C_{29}H_{41}O_7N$. Calculated, C 67.53, H 8.02, 2(OCH₃) 12.04
Found,   " 67.77, " 8.10,   "   11.75

11.493 mg. of dried substance gave 0.58 cc. of CH_4 (30°; 730 mm.).

Found, H 0.197; calculated for 1 H, 0.194

Monomethylanhydro Derivative from Isopyrooxodelphinine—1.65 gm. of pyrooxodelphinine were added to a solution of 66 gm. of zinc chloride in 23 cc. of 5 per cent HCl. The mixture which was kept at 40° formed at first a suspension of partly resinous material, which on stirring gradually dissolved after about 10 minutes, with the exception of a few masses

which had clumped together but on longer manipulation also dissolved. After 1 hour the slightly colored solution was chilled and diluted. The almost clear mixture was extracted continuously in an extractor with chloroform for $1\frac{3}{4}$ hours. The extract on concentration, finally *in vacuo*, to dryness yielded 1.48 gm. of a resinous mass. On solution in a small volume of acetone and seeding, it gradually crystallized as a mass of micro needles or long platelets. 1.01 gm. were collected in the cold with acetone. From dilute acetone it formed micro prisms and rods which melted at 167–170°. Both from acetone and the diluted solvent it separated with solvent and for analysis was dried at 110° and 0.2 mm.

$$[\alpha]_D^{27} = -52° \ (c = 0.90 \text{ of dried substance in 50\% ethanol})$$
$C_{28}H_{31}O_7N$. Calculated, C 68.12, H 6.33; found C 68.16, H 6.25

The concentrated mother liquor from the first crystalline crop, when combined with additional material obtained on continued extraction of the original reaction mixture, gradually crystallized as needles of the completely demethylated substance described below.

In the first demethylation experiments isopyrooxodelphinine was used as the starting material and its complete homogeneity was perhaps somewhat in question. It was found necessary in the initial encounter to fractionate the reaction product.

Thus 1 gm. of isopyrooxodelphinine was treated with zinc chloride in 5 per cent HCl, as described above. The resinous reaction product obtained by continuous extraction with chloroform and concentration was dissolved in a few cc. of methanol. The solution was treated with H_2O as long as a resinous precipitate formed. After standing, the decanted solution (about 20 cc.) was allowed to stand. The next day crystallization had begun and, after rubbing and standing overnight, increased. In the meantime the resinous fraction was redissolved in a small volume of acetone and diluted. After standing, the supernatant solution was decanted and seeded with the above fraction. In this manner a small additional fraction was obtained which was collected with the first crystalline crop and amounted in all to 0.137 gm. On recrystallization from dilute acetone it yielded a first crop (55 mg.) which was contaminated by halogen-containing material. The mother liquor on further manipulation gave a second fraction of needles and platelets (38 mg.) which melted at 168–170° and was halogen-free. It was dried for analysis at 110° and 0.2 mm.

$C_{28}H_{31}O_7N$. Calculated, C 68.12, H 6.33, OCH_3 6.29
Found, " 67.84, " 6.22, " 6.56

The major amount of the reaction material remained in the resinous precipitate and the mother liquors, and this was recombined by chloroform ex-

traction. The material recovered on concentration to a resin was dissolved in a mixture of 5 cc. of chloroform and 5 cc. of benzene and chromatographed through 20 gm. of alumina in benzene. Since the attempt to develop the column with benzene and then with 0.5 per cent methanol in benzene was without result, it was eluted with 1 per cent methanol in benzene. A succession of fractions was obtained and it was found that the first portion eluted contained halogenated substance. The later intermediate and major band crystallized from the diluted acetone solution as characteristic needles and agreed in properties with the fraction described.

$$[\alpha]_D^{27} = -52° \ (c = 0.847 \text{ of dried substance in } 50\% \text{ ethanol})$$
Analysis of Dried Substance—Found, C 67.95, H 6.32, OCH$_3$ 6.40

Later fractions from the chromatogram with 2.5 per cent methanol yielded high melting material which was not further studied.

Octahydromonomethylanhydro Derivative—0.15 gm. of the previous monomethylanhydro derivative was boiled down in methanol to remove acetone of crystallization and the solution in methanol was hydrogenated with 50 mg. of platinum oxide catalyst. Although the process appeared complete after 2 hours, the operation was continued without appreciable further absorption. The absorption in excess of the catalyst requirement was 28 cc., or about 4 moles. After considerable manipulation the substance was obtained from methanol-ether as delicate micro needles or rosettes which melted at 146–147.5° and contained solvent.

For analysis it was dried at 110° and 0.2 mm.

$C_{28}H_{39}O_7N$. Calculated, C 67.02, H 7.84; found, C 66.90, H 7.73

Desmethylanhydroisopyrooxodelphinine—0.1 gm. of the above monomethyl derivative was treated with a solution of 4 gm. of zinc chloride in 1.4 cc. of 5 per cent HCl and heated at 60° for 1 hour. The initial cheesy mass gradually dissolved on manipulation with a rod. The slightly colored solution was diluted and continuously extracted with chloroform. The latter yielded on concentration a gelatinous mass which was further dried *in vacuo*. A solution of the residue in acetone readily crystallized as a mass of delicate needles which melted at 299–301° and contained solvent. Analysis showed it to be methoxyl-free.

For analysis it was dried at 110° and 0.2 mm.

$C_{27}H_{29}O_7N$. Calculated, C 67.61, H 6.10; found, C 67.72, H 6.31

This substance was readily obtained in a larger amount by the direct use of pyrooxodelphinine. 2 gm. of the latter, when heated at 60° in

a solution of 80 gm. of zinc chloride in 28 cc. of 5 per cent HCl, gradually dissolved. After 1 hour the clear yellow solution was diluted and extracted in a funnel four times with 40 cc. portions of chloroform. The latter yielded on concentration 0.49 gm. of a mixture. The aqueous phase was then continuously extracted with chloroform for 18 hours, during which partly gelatinous material separated in the lower flask of accumulated boiling solvent. The latter on concentration, finally *in vacuo*, to dryness yielded 1.3 gm. of partly crystalline material. This was gradually dissolved by refluxing in a large volume (about 700 cc.) of acetone, and, on concentration to about 200 cc., some gelatinous material began to appear but was soon followed by delicate needles. After standing overnight the solid was collected with dry acetone. 0.76 gm. of substance which melted at 299–300° was obtained.

$$[\alpha]_D^{25} = -48.5° \ (c = 0.89 \text{ of dried substance in } 50\% \text{ ethanol})$$
$$\text{Found, C } 67.22, \text{ H } 6.24$$

Material obtained by continued extraction of the aqueous phase and the above acetone mother liquor yielded an additional amount of the same substance and also a fraction of the intermediate monomethyl derivative.

The 0.49 gm. fraction of more readily extracted material, when dissolved in a small volume of chloroform, deposited leaflets which were collected with chloroform. The substance gradually softened to a melt from 240–249° and contained chlorine. The methoxyl content proved negligible.

For analysis the substance was dried at 110° and 0.2 mm.

$$C_{27}H_{28}O_6NCl. \quad \text{Calculated.} \quad C \ 65.10, \ H \ 5.67, \ Cl \ 7.12$$
$$\text{Found.} \quad \text{" } 64.42, \text{ " } 5.94, \text{ " } 6.92$$

0.2 gm. of the substance $C_{30}H_{37}O_8N$ obtained from pyro-α-oxodelphinine with HNO_3 (1) was similarly heated for 45 minutes at 60° with zinc chloride solution. The somewhat colored solution on dilution caused some precipitation of resin and was shaken out four times with about 50 cc. of chloroform. The dried extract on concentration yielded 0.11 gm. of a resin. This consisted mostly of intermediate demethylation products and halogen-containing material and was not further investigated.

The remaining aqueous phase was continuously extracted with chloroform for 20 hours and yielded partly gelatinous material which, when dried, weighed 66 mg. This yielded from acetone a first crop of 41 mg. of delicate needles which melted at 295–296.5°. Analysis of the dried substance gave

$$C_{27}H_{29}O_7N. \quad \text{Calculated, C } 67.61, \text{ H } 6.10; \text{ found, C } 67.20, \text{ H } 6.09$$

Desmethylanhydroketone, $C_{27}H_{27}O_7N$—0.15 gm. of desmethylanhydroiso-pyrooxodelphinine was dissolved in a mixture of 2.3 cc. of acetic acid and 0.75 cc. of H_2O and treated with 0.3 cc. of the Kiliani CrO_3 solution. Oxidation gradually proceeded at room temperature and the reagent was used up in about 1.5 hours. The diluted mixture, after continuous extraction with chloroform, yielded 0.14 gm. of resin. It crystallized from acetone as long thin needles which contained solvent and melted at 191–194°. It is appreciably soluble in water.

For analysis it was dried at 110° and 0.2 mm.

$C_{27}H_{27}O_7N$. Calculated, C 67.89, H 5.70; found, C 67.76, H 6.02

The *oxime* was prepared by allowing the substance to react with a mixture of hydroxylamine hydrochloride and sodium acetate in sufficient 50 per cent methanol. After several days standing and the removal of methanol, material separated which was not definitely crystalline. On dilution of the warm solution in ethanol it separated as a fine micro crystalline powder which melted at 245–248°. The anhydrous substance yielded

$C_{27}H_{28}O_7N_2$. Calculated, C 65.82, H 5.73, N 5.69
Found,   " 65.82, " 6.18 " 5.38

Desmethylanhydroisopyrooxodelphonine—1 gm. of desmethylisopyrooxo-delphinine was suspended in 10 cc. of 70 per cent ethanol and treated with 4 cc. of 0.5 N NaOH. The substance gradually dissolved but there remained in suspension some apparently amorphous material. After 50 minutes the turbid mixture was somewhat diluted and carefully acidified to Congo red with H_2SO_4. After repeated extraction with ether to remove benzoic acid and ester, the aqueous phase was treated with several volumes of absolute ethanol and filtered. The filtrate was carefully neutralized with dilute NaOH to phenolphthalein and concentrated *in vacuo* to dryness. The residue was extracted with absolute ethanol and filtered from Na_2SO_4. The filtrate on concentration gave 0.8 gm. of residue. On treating with acetone-water (4:1) it dissolved on warming and crystallized on seeding as a thick pap. The first fraction collected with the same solvent was 0.28 gm. The mother liquor yielded successive fractions of 0.19 gm., 40 mg., and 22 mg. It formed needles which contained a solvent and melted at 168–170°. It was difficult to obtain the substance entirely ash-free. Attempts to sublime it at 0.03 mm. were unsuccessful, except for a relatively very small amount which flashed over apparently with solvent with the bath at about 120°. This substance melted at 171–173°. When the bath was further heated to 210°, decomposition in the unsublimed residue occurred. Molecular sublimation was

not tried. The substance is readily soluble in H_2O, methanol, and ethanol, and insoluble in ether.

For analysis it was dried at 110° and 0.2 mm.

$C_{20}H_{25}O_6N$. Calculated. C 63.96, H 6.72
Found. (a) " 63.57, " 6.73
(b) " 63.76, " 6.83

Desmethylanhydrodihydroisopyrooxodelphonine—0.1 gm. of desmethylanhydroisopyrooxodelphonine in 50 per cent ethanol with 50 mg. of platinum oxide catalyst absorbed about 1 mole of H_2 in excess of the catalyst requirement within 25 minutes and no further absorption appeared to occur. The filtrate from the catalyst on concentration to dryness yielded a resin which was dissolved in acetone-water (9:1). When crystallization once began after long standing, it separated readily as a powder of glistening minute micro crystals which were solvent-free. It crystallized also from methanol in a similar form. The substance melts at 290–292° and is soluble in H_2O but very sparingly in absolute ethanol, methanol, and acetone.

$C_{20}H_{27}O_6N$. Calculated. C 63.62, H 7.21
Found. (a) " 63.34, " 6.92
(b) " 63.93, " 7.31

The yield of this product was somewhat less than half of the starting material. The mother liquor contained an appreciable amount of more soluble, possibly stereoisomeric, substance which was not crystallized.

All analytical data have been obtained by Mr. D. Rigakos of this laboratory.

SUMMARY

Although instances of partial demethylation of the methoxyl groups of certain delphinine derivatives were previously noted, controlled partial and complete demethylation has now been accomplished with the unsaturated isopyrooxodelphinine. This readily yields with zinc chloride and HCl a monomethyl- or a desmethylanhydro derivative with an accompanying loss of 1 mole of water. In the case of the hydrogenated derivatives, demethylation occurs most readily to the dimethyl stage, with an accompanying loss of 1 mole of water. The last and most resistant methoxyl group involves a secondary hydroxyl which can be oxidized to carbonyl after demethylation.

The isomerization of pyro-α-oxodelphinine to isopyrooxodelphinine has been shown to involve the double bond produced on pyrolysis of α-oxodelphinine with loss of acetic acid.

BIBLIOGRAPHY

1. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **136,** 303 (1940).
2. Jacobs, W. A., and Sato, Y., *J. Biol. Chem.*, in press.
3. Jacobs, W. A., and Huebner, C. F., *J. Biol. Chem.*, **170,** 209 (1947).
4. Craig, L. C., Michaelis, L., Granick, S., and Jacobs, W. A., *J. Biol. Chem.*, **154,** 293 (1944).
5. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **128,** 431 (1939).

A NEW SYNTHESIS OF PHOSPHORYL-ENOLPYRUVIC ACID

BY ERICH BAER AND HERMANN O. L. FISCHER*

(From the Banting and Best Department of Medical Research, Banting Institute, University of Toronto, Toronto, Canada)

While investigating the enzymatic conversion of D-glyceric acid-3-phosphoric acid to pyruvic acid and phosphoric acid, an essential step both in glycolysis and fermentation, Meyerhof and Lohmann (1) observed the formation of a hitherto unknown organic phosphoric acid ester. They succeeded in isolating the new intermediary compound by virtue of the ease of crystallization of the silver-barium salt, and established its constitution as that of phosphoryl-enolpyruvic acid (PEPA). The presence of optical activity in the isolated product thus led Meyerhof and Lohmann to propose and favor a formula (B) which differed from the anticipated structure (A). They realized, of course, the possibility that the activity observed could have been due to contamination by an optically active substance.

$$CH_2{=}C{-}COOH \qquad\qquad CH_2{=}C \underset{O}{\overset{OH}{\underset{\diagdown\diagup}{\overset{\diagup}{{-}{-}\overset{*}{C}\diagdown}}}} OPO_3H_2$$

$$OPO_3H_2$$

$$\text{(A)} \qquad\qquad\qquad\qquad \text{(B)}$$
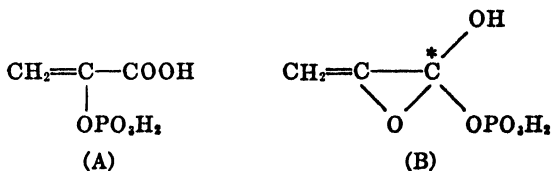
The synthesis of compound A, accomplished by Kiessling (2) in Meyerhof's laboratory, by the interaction of pyruvic acid and phosphorus oxychloride in quinoline, produced a substance which proved to be identical with the natural product and thus established its constitution.

Kiessling's synthesis produced the PEPA, however, in a yield of at best 3 to 5 per cent. In view of the current biochemical interest in this substance a more practical method of preparation was desirable. Despite the apparent simplicity of the molecule, the development of an efficient synthesis of PEPA has proved to be a difficult problem. Various approaches over a period of years have been investigated in this laboratory, and the method which was finally developed is illustrated in the accompanying reaction scheme. Although the new procedure produces the compound in a yield several times that obtained by Kiessling's method and in a higher state of purity, it cannot be regarded as completely

* Present address, Virus Laboratory, Forestry Building, University of California, Berkeley 4, California.

satisfactory. However, no better method of preparing this substance is known at the present time.

In its present form the synthesis is as follows: β-Chlorolactic acid (I) is phosphorylated by means of phosphorus oxychloride in the presence of dimethylaniline. The phosphorylation product, a mixture of organic phosphoric acid esters, is not isolated but is treated immediately with an ethanolic solution of potassium hydroxide to remove the elements of hydrochloric acid from the phosphoryl-β-chlorolactic acid (II). The PEPA thus formed is precipitated, together with other organic phosphates, as the ethanol-insoluble barium salt; it is finally isolated by means of its silver salt (III) and its characteristic silver-barium salt (IV) (1).

The silver-barium salt, after purification by precipitation from dilute nitric acid, is free from inorganic phosphate and other esters of phosphoric acid, gives the correct analysis for $C_3H_2O_6PBaAg + 2H_2O$, and contains pyruvic acid, phosphoric acid, silver, and barium in the theoretical ratios of $1:1:1:1$. Its mercuric acetate-labile phosphorus is identical with the total organic phosphorus, a characteristic of PEPA (1). In alkaline solution the substance consumes 6 moles of iodine, producing 1 mole of iodoform and 1 mole of inorganic phosphate. On acid hydrolysis it forms quantitatively pyruvic acid and phosphoric acid, the rate of hydrolysis being identical with that reported for the natural compound (1).

The crystal form of the silver-barium salt of PEPA varies somewhat with the conditions of crystallization, but the most typical form and arrangement are shown in Fig. 1.

$$
\begin{array}{ccc}
\text{CH}_2\text{Cl—CH—COOH} & \xrightarrow[\text{aniline}]{\text{POCl}_3 \atop \text{Dimethyl-}} & \left[\text{CH}_2\text{Cl—CH—COOH}\right] \\
\qquad\qquad | & & \qquad\qquad | \\
\qquad\text{OH} & & \qquad\text{OPOCl}_2 \\
(I) & & (II)
\end{array}
\xrightarrow[90\%\ \text{C}_2\text{H}_5\text{OH}]{\text{KOH}}
$$

$$
\begin{array}{cc}
\text{CH}_2\text{=C—COOK} & \xrightarrow{\text{Ba(OOCCH}_3)_2} \\
\qquad | & \\
\text{OPO}_3\text{K}_2 &
\end{array}
\quad
\begin{array}{c}
\text{CH}_2\text{=C—COO}\tfrac{1}{2}\text{Ba} \\
\qquad | \\
\text{OPO}_3\text{Ba}
\end{array}
\xrightarrow{\text{AgNO}_3}
$$

$$
\begin{array}{cc}
\text{CH}_2\text{=C—COOAg} & \xrightarrow[\text{Nitric acid}]{\text{Ba(NO}_3)_2} \\
\qquad | & \\
\text{OPO}_3\text{Ag}_2 & \\
(III) &
\end{array}
\quad
\begin{array}{c}
\text{CH}_2\text{=C—COOAg}^* \\
\qquad | \\
\text{OPO}_3\text{Ba} \\
(IV)
\end{array}
$$
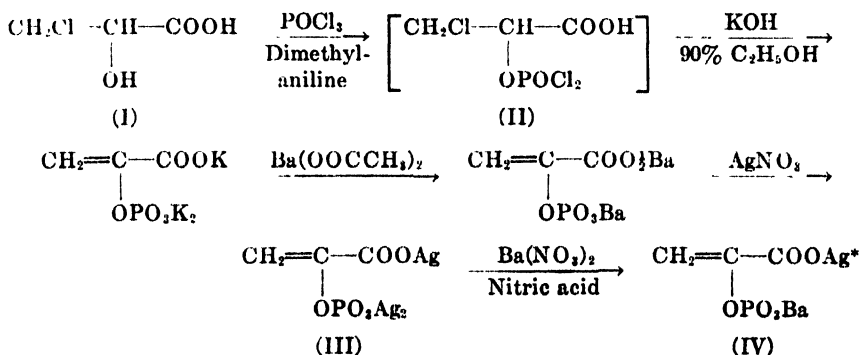
* Placement of the silver and barium in these positions is arbitrary.

The starting material for the synthesis of PEPA, the β-chlorolactic acid, is not at present available commercially. Its preparation by oxidation of epichlorohydrin with nitric acid has been described by von Richter

(3) and Fischer and Leuchs (4). The β-chlorolactic acid thus prepared contains, however, considerable amounts of oxalic acid. In the experimental section of this paper a modified procedure is described for the preparation of pure β-chlorolactic acid by nitric acid oxidation of either epichlorohydrin or glycerol-α-monochlorohydrin.

In view of the greater reactivity of iodo compounds in general, it was thought that the phosphoryl-β-iodolactic acid would, on treatment with alkali, form the PEPA more readily than the corresponding bromo and chloro compounds. Accordingly the investigation was begun with β-iodolactic acid as starting material. The required β-iodolactic acid was prepared from β-chlorolactic acid by halogen exchange as described

Fig. 1. Silver-barium salt of the synthetic phosphoryl-enolpyruvic acid

by Glinsky. The only available abstract of this communication (5) is, however, so lacking in detail that it became necessary to reestablish the conditions of synthesis. The procedure as finally carried out in this laboratory is described in detail below.

Later it was found that the over-all yield of PEPA obtained by direct phosphorylation of β-chlorolactic acid is greater (based on the weight of β-chlorolactic acid) than that obtained by phosphorylation of β-iodolactic acid derived from β-chlorolactic acid. In the event that the β-iodolactic acid becomes more readily available than the corresponding chloro compound, the present procedure for the synthesis of PEPA can be readily adapted to its use as starting material.

Work is in progress in this laboratory on the synthesis of other enol-

phosphates of biological interest by methods similar to that described in this paper.

Phosphoryl-enolpyruvic Acid

Phosphorylation— A solution of 9.96 gm. (0.08 mole) of β-chlorolactic acid (m.p. 78 80°) in 140 ml. of dry dimethylaniline[1] was prepared in a 1 liter three-necked flask equipped with an oil-sealed mechanical stirrer, calcium chloride tube, and dropping funnel. The flask was then immersed in an ice bath and the stirrer set in motion. As soon as the temperature of the solution had reached 2°, a solution of 13.5 gm. (0.088 mole) of freshly distilled phosphorus oxychloride in 60 ml. of dry dimethylaniline was added dropwise over a period of 5 to 6 minutes. 15 minutes after the last of the phosphorus oxychloride had been added, the ice bath was removed and the reaction mixture kept at room temperature for a period of 20 minutes. At the end of this time the speed of the stirrer was increased and a solution of 40 gm. of potassium hydroxide in 400 ml. of 90 per cent ethanol at room temperature was added quickly. The temperature of the mixture rose immediately to 45°. After 2 minutes at this temperature, the flask was immersed in an ice bath and the mixture brought to 30°. The vigorously stirred mixture was maintained at this temperature for 1 hour by placing the flask in a water bath at 30–32°. The suspension was then centrifuged and the precipitate washed at the centrifuge twice with 100 ml. portions of 95 per cent ethanol. The mixture of potassium salts was dissolved in 240 ml. of water and the solution freed of inorganic phosphate by adding ammoniacal magnesia mixture[2] until a test showed that all free phosphate was removed. Approximately 150 ml. of the magnesia mixture were sufficient. An excess was avoided. The ammonium magnesium phosphate was removed by centrifugation and the decanted supernatant liquid mixed with a solution of 48 gm. of barium acetate in 120 ml. of water. The water-insoluble precipitate of barium salts was removed by centrifugation and the decanted supernatant liquid (525 ml.) was diluted gradually with an equal volume of 95 per cent ethanol. After standing for 2 hours at room temperature, the ethanol-insoluble precipitate was centrifuged and washed at the centrifuge consecutively with 150 ml. portions of 95 per cent ethanol, 99 per cent ethanol, and ether.

---

[1] Dried over solid sodium hydroxide.

[2] Prepare as follows: Dissolve 400 gm. of magnesium chloride hexahydrate and 300 gm. of ammonium chloride in 1500 ml. of warm water. Add ammonium hydroxide until the solution is alkaline to litmus. After 1 hour filter the solution and add hydrochloric acid to the filtrate until acid to litmus. Shortly before use 15 ml. of concentrated ammonia per 100 ml. of magnesia mixture are added.

The paste was freed from organic solvent in a stream of air and the resulting fine white powder dried *in vacuo* (0.2 mm.) over phosphorus pentoxide to constant weight.

The mixture of barium salts weighed 13.0 gm. and was free from inorganic phosphate. According to the value for mercuric acetate-labile phosphorus (2.74 per cent) the barium salts contained 1.93 gm. (14.3 per cent) of phosphoryl-enolpyruvic acid. The phosphorus labile to mercuric acetate amounted to only 60.1 per cent of the total organic phosphorus (4.57 per cent). The difference was accounted for by the presence of considerable amounts of other esters of phosphoric acid. The isolation of the pure enol-phosphate was accomplished via the silver and silver-barium salt.

Isolation of Phosphoryl-enolpyruvic Acid As Silver-Barium Salt—The barium salt mixture (13.0 gm.) was dissolved in 250 ml. of 0.2 N nitric acid and the solution was freed from barium ions by adding dropwise 5 N sulfuric acid until, in a centrifuged sample, the presence of a small excess of sulfuric acid could be detected. The barium sulfate was removed by centrifugation and the decanted supernatant liquid mixed with a concentrated aqueous solution of 18.0 gm. of silver nitrate.[3] After the removal of a small precipitate of silver chloride concentrated ammonia was added cautiously drop by drop until the mixture was weakly but distinctly alkaline to litmus, and the voluminous precipitate of silver salts was removed by centrifugation. The well drained silver salts were dissolved in 350 ml. of 0.2 N nitric acid and the solution freed by centrifugation from a small amount of dark brown impurity, added to a solution of 11.0 gm. of barium nitrate in 180 ml. of water. This mixture was quickly poured into 350 ml. of 0.2 N ammonia at room temperature. After 100 ml. of 95 per cent ethanol had been added, the flask was placed in an ice box for a period of at least 3 hours. The colorless silver-barium salt was centrifuged, washed at the centrifuge consecutively with 50 ml. portions of 50 per cent ethanol, 99 per cent ethanol, and ether, and dried *in vacuo* (0.2 mm.) over phosphorus pentoxide for a period of 20 hours.

The white crystalline product weighed 5.3 gm. and gave the following analysis: total P 5.6 per cent; mercuric acetate-labile P 5.2 per cent; organic but not mercuric acetate-labile P 0.2 per cent; inorganic P 0.2 per cent. Theoretical values, total P 6.95 per cent; mercuric acetate-labile P 6.95 per cent.

The silver-barium salt was readily obtained in analytically pure state by reprecipitation from dilute nitric acid.

[3] In order to prevent discoloration of the silver or silver-barium salts all operations from here on must be carried out in subdued light, and the finished preparations should be stored in darkness.

Purification of the Silver-Barium Salt—The crude silver-barium salt (5.3 gm.) was dissolved in 110 ml. of 0.2 N nitric acid and the solution was freed, by centrifugation, of a small amount of brown impurities. To the decanted supernatant were added 13 ml. of a 40 per cent barium acetate solution and the mixture was placed immediately in a dark room for a period of 24 hours. When undisturbed during this time, most of the voluminous precipitate changed into a mass of well formed and dense crystals (see Fig. 1), leaving the impurities in a finely divided and easily suspensible state. The mixture was gently agitated, and, after the heavy material had settled, the supernatant liquid carrying the suspended impurities was decanted. This flotation process was repeated twice with 10 ml. portions of 50 per cent ethanol. Finally the crystalline material was transferred, with 20 ml. of 50 per cent ethanol, to a Büchner funnel, washed with 40 ml. of 99 per cent ethanol, and dried *in vacuo* (10 mm.) at room temperature over phosphorus pentoxide for a period of 18 hours. The colorless silver-barium salt of phosphoryl-enolpyruvic acid weighed 3.6 gm. (10 per cent of the theoretical amount) and was analytically pure.
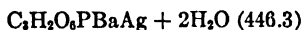
$C_3H_2O_6PBaAg + 2H_2O$ (446.3)
Calculated. C 8.07, H 1.35, P (total organic) 6.95, P (mercuric acetate-labile) 6.95,
    Ag 24.17, Ba 30.78
Found. C 8.31, H 1.29, P (total organic) 6.96, P (mercuric acetate-labile) 6.96,
    Ag 24.08, Ba 30.80

Oxidation with Hypoiodite—A solution of PEPA, prepared by triturating 129.4 mg. of silver-barium salt with dilute hydrochloric acid and removing the silver chloride by centrifugation, was mixed with 25.0 ml. of 0.100 N iodine solution and made alkaline to litmus. After standing 30 minutes, the solution was acidified and the remaining iodine was determined by titration with 0.100 N sodium thiosulfate solution. The iodine consumed amounted to 17.0 ml. of 0.100 N solution; *i.e.*, 97.7 per cent of the amount calculated for 6 atoms of iodine.

Determination of Pyruvic Acid—A dilute solution of PEPA, obtained by triturating 150.4 mg. of silver-barium salt with three 5 ml. portions of 2.5 N hydrochloric acid and removal of the silver chloride by centrifugation, was mixed with a warm solution of 250 mg. of 2,4-dinitrophenylhydrazine in 15 ml. of 2.5 N hydrochloric acid, and the mixture was kept in a boiling water bath for a period of 90 minutes. After standing for 2 hours in an ice box at 6° the hydrazone was collected by suction on a crucible with a fritted glass disk, washed with dilute hydrochloric acid and water, and dried *in vacuo* (0.2 mm.) over phosphorus pentoxide. The weight of the precipitate was 90.0 mg. The crucible was washed

with dilute sodium bicarbonate solution and water until the filtrate was colorless, dried, and weighed as before. The loss in weight, equal to the amount of 2,4-dinitrophenylhydrazone of pyruvic acid, was 89.0 mg. or 97.9 per cent of the theoretical value.

The sodium bicarbonate solution, on acidification, yielded pyruvic acid 2,4-dinitrophenylhydrazone, which after recrystallization from 99 per cent ethanol, melted with decomposition at 218–219°. The melting point of an authentic sample of pyruvic acid 2,4-dinitrophenylhydrazone was 218–219° (decomposition).

Acid Hydrolysis of Phosphoryl-enolpyruvic Acid—To prepare a solution of PEPA in hydrochloric acid, 37.3 mg. of the silver-barium salt were triturated three times with 10 ml. portions of 1 N hydrochloric acid. After each trituration the solid was centrifuged, the supernatant liquid decanted, and the precipitate resuspended in hydrochloric acid. The supernatant solutions were combined and the volume brought to 50 ml. with 1.0 N hydrochloric acid. A series of stoppered Pyrex test-tubes, each containing 2.0 ml. of the PEPA solution (1.67×10^{-3} M), was kept in a strongly boiling water bath. At intervals of 5 minutes tubes were removed and their inorganic phosphorus determined colorimetrically. The rate of hydrolysis was found to be identical with that reported for the natural PEPA. Both rates of hydrolysis are plotted in Fig. 2.

β-Chlorolactic Acid

To 440 ml. of cold nitric acid (sp. gr. 1.42) in a 5 liter round bottomed flask surrounded by an ice bath, 110.5 gm. (1.0 mole) of glycerol-α-mono-chlorohydrin were added from a separatory funnel over a period of 10 minutes. The flask was then placed on a boiling water bath[4] and its contents heated until reaction set in, as indicated by the appearance of gas bubbles. The water bath was then immediately removed and the reaction allowed to proceed spontaneously. After approximately 10 minutes the vigorous reaction had subsided and the mixture was heated on a boiling water bath for half an hour. The cooled reaction mixture was transferred to a distilling flask and concentrated as far as possible *in vacuo* (8 to 10 mm. of Hg) at a bath temperature not exceeding 55°. The partially crystallized residue was dissolved in 370 ml. of distilled water, the solution was made neutral to litmus with anhydrous sodium carbonate, and the sodium oxalate was removed by filtration with suction. The remainder of the oxalic acid was removed by adding to the filtrate dropwise a concentrated aqueous solution of calcium chloride until no more precipitate formed, and filtering the mixture. Concentrated

---

[4] This reaction is accompanied by the evolution of large amounts of nitrogen oxides and therefore must be carried out under a fume hood.

sulfuric acid, equivalent to the amount of anhydrous sodium carbonate used above, was added slowly and with stirring to the ice-cold filtrate and the solution was extracted six to eight times with 230 ml. of ether. The combined ether extracts were evaporated under diminished pressure and the residue heated *in vacuo* to 60° for 2 hours. The remaining solid was dissolved in 120 ml. of hot *n*-butyl ether and the solution placed in ice for 3 hours. The colorless crystals were collected on a Büchner funnel, washed on the filter with low boiling petroleum ether, and dried *in*
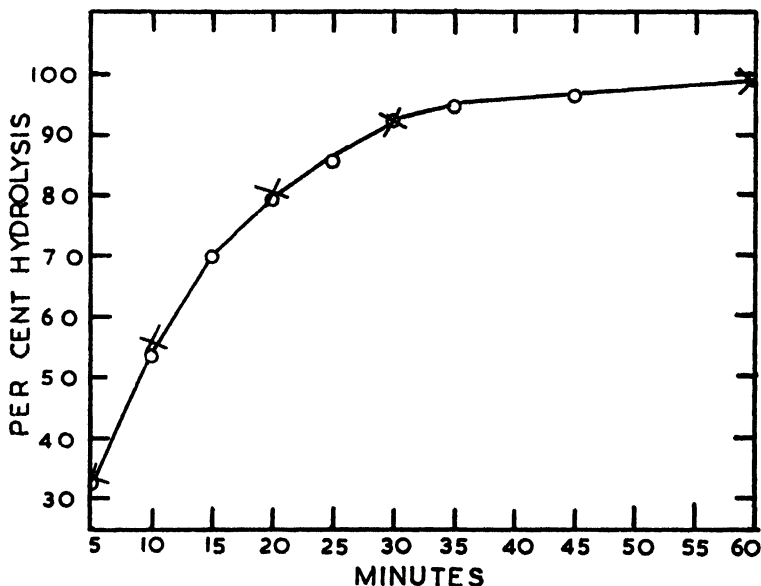
FIG. 2. Rates of hydrolysis of phosphoryl-enolpyruvic acid in 1.0 N hydrochloric acid at 100°. O, synthetic product; X natural product (Meyerhof and Lohmann (1)).

vacuo. The β-chlorolactic acid melted at 78–80° and weighed 38.5 gm. (32 per cent of the theoretical amount).

By substituting an equivalent amount of epichlorohydrin (92.5 gm.) in the above procedure, β-chlorolactic acid of the same purity (m.p. 78.5–80°) and practically the same yield (37.8 gm., 30.3 per cent of the theoretical amount) was obtained.

β-Iodolactic Acid

A solution of 93.4 gm. (0.75 mole) of β-chlorolactic acid and 170 gm. (1.13 mole) of anhydrous sodium iodide[5] in 550 ml. of dry acetone was

[5] Dried in the oven at 120°.

prepared in a 1 liter round bottomed flask with ground glass joint bearing a reflux condenser and a calcium chloride tube. The reaction flask was placed in a water bath of 65° and the solution refluxed for 24 hours. The mixture was then cooled to room temperature, filtered, and the sodium chloride washed on the filter several times with a small amount of dry acetone. The combined filtrates together with 46 gm. (0.31 mole) of dry sodium iodide were refluxed for 24 hours. The cooled solution was filtered, the sodium chloride washed with dry acetone, and the combined filtrates concentrated under reduced pressure to a thick syrup. The partially solid residue was dissolved in 160 ml. of distilled water and the solution decolorized with solid sodium bisulfite. After the addition of 40 ml. of 5 N sulfuric acid the solution was extracted twice with 150 ml. of petroleum ether[6] (b.p. 30–60°) and five times with 130 ml. of peroxide-free ether. The combined ether extracts were dried with anhydrous sodium sulfate, the ether removed under diminished pressure, and the residue was kept for several hours in a vacuum of 10 mm. at a bath temperature of 55°. The dark brown mass was spread on a clay plate to remove oily impurities and then thoroughly dried *in vacuo* over phosphorus pentoxide. For purification the crude β-iodolactic acid, weighing 131 gm., was dissolved in 260 ml. of dry ethyl acetate and reprecipitated by the addition of 560 ml. of carbon tetrachloride. To increase the yield the mixture was placed in a dry ice-acetone bath at −65° for half an hour. The solid was filtered by suction on a Büchner funnel, washed with cold (−30°) carbon tetrachloride until both crystals and filtrate were colorless, and dried *in vacuo*. Yield of pure β-iodolactic acid, 106 gm. (65.5 per cent of the calculated amount); m.p. 99–100° (6).

SUMMARY

1. A new and more efficient synthesis of phosphoryl-enolpyruvic acid (PEPA) is described.

2. The synthesis involves three steps: (*a*) phosphorylation of β-chlorolactic acid with phosphorus oxychloride; (*b*) removal of the elements of hydrochloric acid from phosphoryl-β-chlorolactic acid by treatment with alkali; (*c*) isolation of the phosphoryl-enolpyruvic acid as the silver-barium salt.

3. The properties of the phosphoryl-enolpyruvic acid from β-chlorolactic acid were found to be identical with those reported for the natural PEPA (Meyerhof and Lohmann) and the synthetic PEPA obtained by phosphorylation of pyruvic acid (Kiessling).

---

[6] The petroleum ether extraction removes higher boiling condensation products of acetone.

We wish to express our sincere appreciation to Professor W. Stanley Hartroft, University of Toronto, for his kindness in preparing the photomicrograph.

BIBLIOGRAPHY

1. Meyerhof, O., and Lohmann, K., *Biochem. Z.*, **273**, 60 (1934).
2. Kiessling, W., *Ber. chem. Ges.*, **68**, 597 (1935).
3. von Richter, V., *J. prakt. Chem.*, **20**, 193 (1879).
4. Fischer, E., and Leuchs, H., *Ber. chem. Ges.*, **35**, 3794 (1902).
5. von Richter, V., *Ber. chem. Ges.*, **6**, 1257 (1873); correspondence, Die Chemie auf der vierten russischen Naturforscherversammlung in Kasan (1873).
6. Melikow, *Ber. chem. Ges.*, **14**, 937 (1881).

SYNTHESIS OF PEPTIDES OF METHIONINE AND THEIR CLEAVAGE BY PROTEOLYTIC ENZYMES*

By CHARLES A. DEKKER, STERLING P. TAYLOR, Jr., AND JOSEPH S. FRUTON

(From the Department of Physiological Chemistry, Yale University, New Haven)

In a previous communication from this laboratory (1), a method was described for the enzymatic resolution of DL-methionine. The ready availability of the optically active enantiomorphs has made possible the application of the carbobenzoxy method to the synthesis of peptides of L- and D-methionine. A search of the literature has indicated that the only methionine peptide described thus far is glycyl-L-methionine (2), which was obtained by the treatment of L-methionine with chloroacetyl chloride, followed by the amination of the resulting chloroacetylamino acid. The rotation of the peptide was not reported, however, and, as is noted in the experimental section of this communication, the melting point of this product is about 55° lower than that found for glycyl-L-methionine prepared by the carbobenzoxy method. In what follows, there is described the synthesis of a series of methionine peptides and peptide derivatives, and a number of these have been examined with regard to their susceptibility to the hydrolytic action of several proteolytic enzymes. In addition, the availability of such peptides of methionine makes possible the study of other metabolic reactions involving compounds in which this amino acid is bound in peptide linkage. The results of such studies will be reported in future communications.

Synthesis of Peptides of Methionine—In the experimental section, directions are given for the synthesis of the free peptides glycyl-L- and D-methionine, L- and D-methionylglycine, α-L-glutamyl-L-methionine, L-methionyl-L-methionine, L-methionyl-L-tyrosine, and L-methionylglycyl-glycine. In addition, there have been prepared a number of derivatives of L- and D-methionine, and of peptides containing L- or D-methionine residues. Aside from the procedure for the removal of the carbobenzoxy group, the methods employed in these syntheses were similar to those applied to the preparation of peptides of other amino acids (3), and require no special mention at this point. In view of the presence of the sulfur atom in the methionine side chain, it was to be expected that hy-

drogenolysis of the carbobenzoxy group with palladium black as the catalyst would not be a feasible procedure. It was found, however, that, in the case of most of the methionine derivatives subjected to hydrogenolysis, this could be effected in the usual way with palladium black, although occasionally the time of hydrogenolysis was prolonged, and fresh catalyst had to be added to complete the reaction. More serious difficulties were encountered in the application of this method to the hydrogenolysis of carbobenzoxyglycyl-L-methionine and of carbobenzoxy-L-methionyl-L-methionine, and these compounds were reduced with sodium in liquid ammonia (4). Since this procedure may involve demethylation of the methionine side chain to give derivatives of homocysteine (5), methyl iodide was added at the end of the reaction so as to insure remethylation of the sulfur (6). In this manner, it was possible to obtain analytically pure glycyl-L-methionine and L-methionyl-L-methionine, although the yields were somewhat lower than those usually obtained by catalytic hydrogenolysis with palladium black. In the present study, no attempt was made to isolate the homocysteine peptides formed by the treatment of the methionine peptides with sodium in liquid ammonia. It may be expected that the synthesis of such compounds may be effected more economically by the use of the S-benzyl-L-homocysteine obtained upon acid hydrolysis of carbobenzoxy-L-methioninanilide (1).

In the course of the attempted preparation of carbobenzoxy-L-methionylglycinamide, it was noted that when the corresponding ethyl ester was treated with ammonia in methanol for 2 days at room temperature there was formed, in addition to the expected product, an appreciable quantity of the amide of 5-[2-(methylthio)ethyl]hydantoin-3-acetic acid (I).

$$C_6H_5CH_2OCO-NH-CH-CH_2CH_2SCH_3$$

$$\begin{array}{c} | \\ CO \\ | \\ NH \\ | \\ CH_2 \\ | \\ COOC_2H_5 \end{array} \quad \xrightarrow[\text{CH}_2\text{OH}]{\text{NH}_3}$$

$$CO-NH-CH-CH_2CH_2SCH_3$$

$$\begin{array}{c} | \\ CO \\ | \\ N \\ | \\ CH_2 \\ | \\ CONH_2 \end{array} \quad + \quad C_6H_5CH_2OH$$

(I)
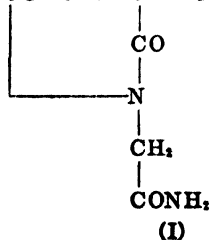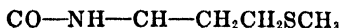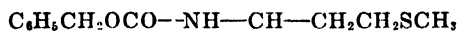
The reaction leading to the formation of (I) is analogous to that previously observed in the attempted amination of carbobenzoxy-L-phenylalanylglycine ethyl ester, which was converted to the amide of 5-benzylhydantoin-3-acetic acid (7). To these two cases of hydantoin formation, with the elimination of benzyl alcohol, may be added the behavior of carbobenzoxy-L-leucylglycine ethyl ester which, on treatment with ammonia in methanol, yields the amide of 5-isobutylhydantoin-3-acetic acid.

TABLE I

Action of Papain and Ficin on Acylamino Acid Amides

Substrate, 0.05 mM per cc. of test solution; papain concentration, 0.16 mg. of protein N per cc. of test solution; ficin concentration, 0.03 mg. of protein N per cc. of test solution; methanol, 0.3 cc. per cc. of test solution; concentration of activator (cysteine), 0.01 M; pH 5.1 to 5.4 (0.2 M citrate buffer); temperature, 38°.

Substrate*	Hydrolysis†					
	Papain‡			Ficin§		
	1 hr.	2 hrs.	24 hrs.	1 hr.	2 hrs.	24 hrs.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Carbobenzoxy-L-methioninamide‖........	17	30		51	72	
Carbobenzoxy-D-methioninamide‖. ...		0	0		1	2
Benzoylglycinamide (10)		6	15		0	0
Carbobenzoxy-L-leucinamide‖ (11).......	10	19	66	15	24	54
Carbobenzoxy-L-serinamide (12)...........		8	42		9	82
Carbobenzoxy-L-threoninamide...		5	25		6	22
Carbobenzoxy-L-isoglutamine (13)		4	39	29	52	
Carbobenzoxy-L-isoasparagine (13).... ...		0	2		3	11
Benzoyl-L-argininamide (14)	28	55		6	11	40
Benzoyl-L-histidinamide (14).		0	0		0	0
Carbobenzoxy-L-phenylalaninamide‖ (7)..		0	6		0	2

* The figures in parentheses are bibliographic reference numbers.

† The rate of enzymatic hydrolysis was determined by microtitration of the carboxyl groups liberated during the experiment.

‡ Prepared according to the procedure described in (15).

§ Merck preparation.

‖ This substance was not completely in solution at the start of the experiment.

It would appear that these reactions are closely related to that postulated in the conversion of carboethoxyglycylglycine ethyl ester to carbonylbisglycine by means of alkali, which has been explained by the assumption that there occurs the formation of hydantoin-3-acetic acid as a transient intermediate (8). This is in accord with the observation that such hydantoin derivatives are readily hydrolyzed by alkali (9).

Hydrolysis of Carbobenzoxy-L-methioninamide (CMA) by Papain and Ficin—It will be noted in Table I that CMA is readily hydrolyzed by

cysteine-activated papain and ficin at pH values near 5. In performing these experiments, methanol (0.3 cc. per cc. of test solution) was added to dissolve some of the substrate, which is very sparingly soluble in water. This insolubility serves to explain the failure to observe appreciable enzymatic hydrolysis in the absence of added methanol. A similar situation has been noted in the case of the action of chymotrypsin on benzoyl-L-tyrosinamide and on benzoylglycyl-L-tyrosinamide (7, 16). Since it is to be expected that, in the presence of 30 per cent methanol, the rate of enzymatic action will be different from that found in aqueous solution, in making a comparison of the relative rates of cleavage of a series of acylamino acid amides by papain and ficin (*cf.* Table I), it seemed desirable to conduct all the experiments in 30 per cent methanol, even if a particular compound was soluble in water. In some cases, the acylamino acid amide was not completely dissolved in 30 per cent methanol at the start of the enzymatic test, and, for this reason, no calculations have been made of rate constants.

A comparison of the rate of hydrolysis of CMA with the rates found for the other acylamino acid amides mentioned in Table I shows the methionine derivative to be among the most sensitive synthetic substrates for papain and ficin. Of especial interest is the finding that, in the case of papain, CMA is hydrolyzed somewhat more slowly than is benzoyl-L-argininamide (BAA), while ficin acts upon CMA much more rapidly than it does on BAA. If it is assumed that the enzymatic components of papain and ficin acting on BAA are homospecific enzymes (17), it would follow that the hydrolysis of CMA is performed by an endopeptidase different from these, and that ficin is a relatively richer source of the CMA-splitting enzyme than is papain. The extreme sensitivity, to papain and ficin, of peptide bonds involving the carbonyl group of a methionine residue focuses attention upon such linkages as possible points of preferential cleavage of proteins by these enzymes.

The rapid hydrolysis of CMA by an enzymatic component of papain is not surprising in view of the previous finding that this enzyme preparation is an effective catalyst in the synthesis of acid amide linkages involving the carbonyl group of a methionine residue (1). This is in accord with the conclusion, drawn from earlier studies (18), that, in the action of proteolytic enzymes, the specificity of hydrolysis is the same as that of synthesis. Also, as might have been expected from the stereochemical specificity of the enzymatic synthesis of methionine derivatives by papain (1), this enzyme preparation showed no appreciable action on carbobenzoxy-D-methioninamide. A similar negative result was noted with ficin.

Despite the earlier findings that activated papain hydrolyzes benzoylglycinamide, carbobenzoxy-L-isoglutamine (CIG) (19), carbobenzoxy-L-

serinamide (12), carbobenzoxy-L-leucinamide (20), and BAA (14), and that activated ficin splits BAA (21), to date there have been no systematic studies of the action of these enzyme preparations on an extensive series of these and of other acylamino acid amides. For this reason, the data in Table I, which include, in addition to the amino acid derivatives mentioned above, the acylamino acid amides derived from L-histidine, L-threonine, L-phenylalanine, and L-aspartic acid, are of interest in connection with the specificity of the enzymatic components of papain and ficin. It will be seen that carbobenzoxy-L-threoninamide is hydrolyzed by these enzyme preparations at rates that are similar to those noted for the serine analogue. In contrast, however, the closely related carbobenzoxy-L-isoasparagine and CIG differ widely in their sensitivity to enzyme action, the former being much more resistant to the action of papain or ficin.

As was indicated above, the data in Table I suggest that the hydrolysis of CMA and of BAA are performed by different enzymatic components of papain. Evidence has been adduced previously (22) to show that the hydrolysis of BAA and of CIG by papain preparations is performed by different enzymatic components. It remains a task of the future to determine whether the hydrolysis of the other acylamino acid amides mentioned in Table I involves the action of still other enzymes present in papain preparations. Similar considerations apply to the question of the enzymatic heterogeneity of ficin preparations. Furthermore, no statement can be made at present as to the relative stability of the various enzymatic components of papain and ficin in the presence of the methanol concentrations used in these studies.

Hydrolysis of Methionine Derivatives by Carboxypeptidase—Previous work (23, 24) has shown that pancreatic carboxypeptidase acts at peptide linkages adjacent to a free carboxyl group in substrates of the following general formula, in which the dotted line denotes the place of enzymatic cleavage.

$$R \qquad R'$$

$$C_6H_5CH_2OCO—NHCHCO\!\!+\!\!NHCHCOOH$$

It has been found that when R is a hydrogen atom, the most rapid hydrolysis by carboxypeptidase is attained with substrates in which R' is the side chain of L-phenylalanine. The introduction of other side chains in place of that of phenylalanine leads to a progressively slower rate of hydrolysis, in the order tyrosine, leucine, isoleucine, alanine, glycine (24). More recent studies (25) have shown that tryptophan falls between tyrosine and leucine in this series. When the nature of R is varied, and R'

is kept the same, the substitution of the methylene group of glycine by the side chain of L-alanine or of L-glutamic acid leads to a decrease in the rate of hydrolysis (23), while the introduction of the side chain of L-tryptophan causes a 3-fold increase in the rate of enzyme action (25).

It has been postulated (23) that the quotient of the rates of hydrolysis of two substrates differing only with respect to the R group is independent of the nature of the R′ groups, when these are identical in the two substrates compared. Support for this view has come from data published previously (23, 25) and it seemed of interest to determine whether the

<div align="center">TABLE II</div>

<div align="center">Action of Crystalline Carboxypeptidase* on Synthetic Substrates</div>

Substrate concentration, 0.05 mM per cc. of test solution; pH 7.6 to 7.7 (0.1 M veronal buffer); temperature, 25°.

Substrate†	Enzyme, protein N per cc. test solution	K‡	C§
	mg.		
Carbobenzoxyglycyl-L-phenylalanine (26)	0.0003	0.0038	12.7
Carbobenzoxyglycyl-L-tyrosine (27)	0.0006	0.0042	7.0
Carbobenzoxyglycyl-L-methionine	0 003	0.0036	1.2
Carbobenzoxy-L-glutamyl-L-phenylalanine (28)	0.006	0.0046	0.78
Carbobenzoxy-L-glutamyl-L-methionine	0.015	0.0011	0.073
Carbobenzoxy-L-methionyl-L-tyrosine	0.0006	0.0047	7.8
Carbobenzoxy-L-methionyl-L-methionine	0.0015	0.0021	1.4
Carbobenzoxyglycyl-D-methionine	0.015	0.0000	

* Four times crystallized preparation.

† The figures in parentheses are bibliographic reference numbers.

‡ $K = \dfrac{1}{\text{min.}} \log \dfrac{100}{100 - \% \text{ hydrolysis}}$ (apparent first order constant; hydrolysis determined by titration of liberated carboxyl groups).

§ $C = \dfrac{K}{\text{mg. protein N per cc. test solution}}$ (proteolytic coefficient (21)).

hypothesis would hold for peptides in which R or R′, or both, represented the side chain of methionine.

It will be seen from Table II that the replacement of the side chain of phenylalanine in carbobenzoxyglycyl-L-phenylalanine (CGP) by that of methionine leads to a slower rate of hydrolysis, which is approximately one-tenth of that for CGP. A similar ratio is found on comparison of the rates of hydrolysis of carbobenzoxy-L-glutamyl-L-methionine and of carbobenzoxy-L-glutamyl-L-phenylalanine. Similarly, carbobenzoxyglycyl-L-tyrosine (CGT) is hydrolyzed about 6 times as fast as carbobenzoxyglycyl-L-methionine (CGM), and this quotient is also observed for the

relative rates of hydrolysis of carbobenzoxy-L-methionyl-L-tyrosine (CMT) and of carbobenzoxy-L-methionyl-L-methionine (CMM). Furthermore, comparison of the relative rates of hydrolysis of CGT and of CMT shows that the introduction of the methionine side chain as the R group does not alter the rate of carboxypeptidase action appreciably. This is the result also noted on comparison of the rates for CGM and CMM. It would appear, therefore, that the data on the methionine-containing substrates of carboxypeptidase are in general accord with the previous studies on the effect of substitution of side chain groups in substrates of this enzyme.

<div align="center">TABLE III</div>

<div align="center">*Action of Crystalline Pepsin* on Synthetic Substrates*</div>

Substrate, 0.05 mM per cc. of test solution; concentration of pepsin, 1.2 mg. of protein N per cc. of test solution; pH 4.0 to 4.1 (0.2 M acetate buffer); temperature, 38°.

Substrate	Hydrolysis in 24 hrs.[†]
	per cent
Carbobenzoxy-L-glutamyl-L-tyrosine[‡] (28)[§]	51
Carbobenzoxy-L-methionyl-L-tyrosine[‡]	35
L-Methionyl-L-tyrosine	25[‖]

* Armour preparation.
† Determination of liberated amino N by Van Slyke nitrous acid method.
‡ This substance was not completely in solution at the start of the experiment.
§ Bibliographic reference number.
‖ After 48 hours, tyrosine crystallized from the solution.

In agreement with earlier studies on the antipodal specificity of carboxypeptidase, carbobenzoxyglycyl-D-methionine was not split by this enzyme under the experimental conditions employed in this study.

Action of Pepsin on Methionine Derivatives—Among the first synthetic substrates found for proteinases was carbobenzoxy-L-glutamyl-L-tyrosine, which is hydrolyzed by crystalline pepsin at pH 4 with the formation of carbobenzoxy-L-glutamic acid and of L-tyrosine (28). It was shown that replacement of the glutamyl residue by that of glycine (28) or of cysteine (29) did not abolish the enzymatic action. It was to be expected, therefore, that CMT would also be hydrolyzed by pepsin, and this was found to be the case (*cf.* Table III). Of especial interest was the finding that L-methionyl-L-tyrosine (MT) is hydrolyzed by crystalline pepsin. It has been shown by Harington and Pitt Rivers (29) that dipeptides such as L-cysteinyl-L-tyrosine are split by this enzyme, and they concluded that the previous views concerning the specificity of pepsin (18) required re-

vision. The fact that MT is a substrate for pepsin supports this conclusion, and emphasizes the necessity for a renewed study of the specificity of this enzyme.

Action of Swine Kidney Extract on Methionine Peptides—A preliminary examination has been made of the hydrolysis of several methionine peptides by an aqueous extract of desiccated swine kidney cortex. The data in Table IV show that all the peptides of L-methionine tested were rapidly hydrolyzed by this enzyme preparation. It will be noted that the rates of hydrolysis of L-methioninamide (MA), L-methionylglycine (MG), and

TABLE IV

Hydrolysis of Methionine Peptides by Aqueous Extract of Swine Kidney Cortex[*]

Substrate concentration, 0.05 mM per cc. of test solution; enzyme concentration, 0.032 mg. of protein N per cc. of test solution; pH 7.4 to 7.8 (0.1 M veronal buffer); temperature, 38°.

Substrate[†]	Hydrolysis[‡]			
	No added activator		+ 0 001 M MnSO₄	
	1 hr.	2 hrs.	1 hr.	2 hrs
	per cent	*per cent*	*per cent*	*per cent*
L-Leucinamide acetate (11)	5	15	15	50
L Methioninamide acetate		5	8	26
L-Leucylglycine	33	58	58	84
L-Methionylglycine	17	37	55	84
L-Leucylglycylglycine (30)	20	41	35	82
L-Methionylglycylglycine	6	13	21	56
Glycyl-L-leucine	49			
Glycyl-L-methionine	54			
L-Glutamyl-L-phenylalanine (31)	10	17		
L-Glutamyl-L-methionine	17	30		

* 2 gm. of desiccated swine kidney cortex (Viobin) were stirred with 20 cc. of water for 30 minutes at room temperature. The suspension was filtered by gravity, and the filtrate was used for the enzyme experiments.

† The figures in parentheses are bibliographic reference numbers.

‡ Determined by titration of liberated carboxyl groups.

L-methionylglycylglycine (MGG) are accelerated by the addition of 0.001 M MnSO₄. For purposes of comparison, the action of the swine kidney extract on the corresponding derivatives of L-leucine is included in Table IV, and it will be seen that there is considerable similarity in the behavior of the two sets of compounds. As shown previously (32), the activation of L-leucine aminopeptidase by manganese ions is a time reaction; this is indicated in Table IV by the more rapid hydrolysis of the leucyl derivatives during the 2nd hour of the incubation period than at the start of the experiments. A similar result is noted for the hydrolysis of MA, MG,

and MGG. These findings raise the possibility that the side chain specificity of swine kidney leucine aminopeptidase may not be as discriminating as is indicated by the name assigned to this enzyme and that it can also hydrolyze peptides of L-methionine and of other amino acids. This question cannot be answered unequivocally, however, without further progress in the purification of the individual peptidases of swine kidney.

Table IV also includes data on the hydrolysis of glycyl-L-methionine and of L-glutamyl-L-methionine by the swine kidney extract. The observed rates are similar to those noted for the comparable peptides in which the L-leucine residue has replaced that of L-methionine.

<div align="center">EXPERIMENTAL</div>

L-*Methionine Methyl Ester Hydrochloride*—20 gm. (0.134 mole) of L-methionine ($[\alpha]_D^{25} = -21.0°$; 2 per cent in 0.2 N hydrochloric acid) were suspended in 200 cc. of methanol and esterified with dry HCl in the usual manner. After removal of excess HCl and of methanol under reduced pressure, there remained a solid residue which was recrystallized from methanol-ether. Yield, 23 gm.; m.p., 151°.

<div align="center">$C_6H_{14}O_2NSCl$ (199.7). Calculated, N 7.0; found, N 7.1</div>

D-*Methionine Methyl Ester Hydrochloride*—This compound was prepared from D-methionine ($[\alpha]_D^{25} = +20.8°$; 2 per cent in 0.2 N hydrochloric acid) in the same manner as the L isomer. M.p., 149–150°.

<div align="center">$C_6H_{14}O_2NSCl$ (199.7). Calculated, N 7.0; found, N 6.9</div>

DL-*Methionine Methyl Ester Hydrochloride*—This compound was prepared from DL-methionine in the same manner as the L isomer. M.p., 109–111°.

<div align="center">$C_6H_{14}O_2NSCl$ (199.7). Calculated, N 7.0; found, N 6.8</div>

Carbobenzoxy-L-*methioninhydrazide*—15 gm. (0.075 mole) of L-methionine methyl ester hydrochloride were dissolved in 20 cc. of water and 200 cc. of ethyl acetate were added. The free ester was formed by the addition of an excess of a saturated potassium bicarbonate solution, and 15 cc. of carbobenzoxy chloride were then added. The excess carbobenzoxy chloride was destroyed with pyridine, and the ethyl acetate layer was washed successively with water, dilute hydrochloric acid, and water, and dried over Na_2SO_4. Removal of the solvent *in vacuo* left an oil which was dissolved in 150 cc. of absolute ethanol, and to the solution were added 5.7 cc. of hydrazine hydrate. The reaction mixture was left at room temperature for 56 hours, and the solution was then concentrated under reduced pressure, leaving a crystalline product which was recrystallized from methanol-water. Yield, 17.5 gm.; m.p., 110–112°.

$C_{13}H_{19}O_2N_3S$ (297.4). Calculated, N 14.1; found, N 14.2 (Dumas)

Carbobenzoxy-D-methioninhydrazide—This compound was prepared in the same manner as the L isomer. M.p., 110–112°.

$C_{13}H_{19}O_2N_3S$ (297.4). Calculated, N 14.1; found, N 14.0 (Dumas)

Carbobenzoxy-DL-methioninhydrazide—This compound was prepared in the same manner as the L isomer. M.p., 107–108°.

$C_{13}H_{19}O_2N_3S$ (297.4). Calculated, N 14.1; found, N 14.3 (Dumas)

Carbobenzoxy-L-methioninamide—The syrupy carbobenzoxy-L-methionine methyl ester (from 4 gm. of L-methionine) was dissolved in 100 cc. of methanol previously saturated with dry ammonia at 0°. The solution was kept at room temperature for 60 hours, and then concentrated under reduced pressure, yielding a crystalline product which was recrystallized from ethanol-water. Yield, 5.7 gm.; m.p., 125°.

$C_{13}H_{18}O_3N_2S$ (282.3). Calculated, N 9.9; found, N 9.9

Carbobenzoxy-D-methioninamide—This compound was prepared in the same manner as the L isomer. M.p., 125°.

$C_{13}H_{18}O_3N_2S$ (282.3). Calculated, N 9.9; found, N 9.9

Carbobenzoxy-DL-methioninamide—This compound was prepared in the same manner as the L isomer. M.p., 119–121°.

$C_{13}H_{18}O_3N_2S$ (282.3). Calculated, N 9.9; found, N 9.9

L-Methioninamide Acetate—2 gm. (7.1 mM) of carbobenzoxy-L-methioninamide were dissolved in a mixture of 25 cc. of methanol and 1 cc. of glacial acetic acid, and were subjected to hydrogenolysis with palladium black as the catalyst. After 3 hours, the catalyst became inactive. The reaction mixture was decanted into a flask containing fresh palladium black, and the hydrogenolysis was continued for an additional 4 hours. The catalyst was then removed by filtration and washed with methanol and hot water. Concentration of the filtrate and washings *in vacuo* brought about the crystallization of unchanged carbobenzoxy-L-methioninamide (0.25 gm.) which was removed by filtration. The filtrate was concentrated to dryness, and the residue was recrystallized from hot ethyl acetate. Yield, 1.25 gm.; m.p., 103°.

$C_7H_{16}O_2N_2S$ (208.3). Calculated, N 13.5; found, N 13.5

D-Methioninamide Acetate—This compound was prepared in the same manner as the L isomer. M.p., 101–102°.

$C_7H_{16}O_2N_2S$ (208.3). Calculated, N 13.5; found, N 13.5

Carbobenzoxyglycyl-L-methionine—To a dry ethereal solution of carbobenzoxyglycyl chloride (from 11.5 gm. of carbobenzoxyglycine) there were added at 0° an ethereal solution of L-methionine methyl ester (from 8.0 gm. of the hydrochloride) and 75 cc. of a saturated aqueous solution of potassium bicarbonate. The mixture was shaken at 0° for 30 minutes, and then was left at room temperature for 1 hour (with occasional shaking). Pyridine was then added to destroy any remaining chloride. The ether layer was separated and washed successively with water, hydrochloric acid, water, potassium bicarbonate solution, and water, and was then dried over Na_2SO_4. After evaporation of the solvent under reduced pressure, an oily residue (8.4 gm.) remained which could not be crystallized. The oil was dissolved in 100 cc. of 95 per cent ethanol, and 25 cc. of N NaOH were added. After 30 minutes, the solution was acidified to pH 4 with N hydrochloric acid and the ethanol was removed *in vacuo*. Upon chilling the solution, crystals were obtained. Yield, 7.2 gm. After recrystallization from ethyl acetate-petroleum ether, the substance melted at 110–111°.

$C_{15}H_{20}O_5N_2S$ (340.4). Calculated, N 8.2; found, N 8.1

Carbobenzoxyglycyl-D-methionine—This compound was prepared in the same manner as the L isomer. M.p., 107–109°.

$C_{15}H_{20}O_5N_2S$ (340.4). Calculated, N 8.2; found, N 8.1

Glycyl-L-methionine—680 mg. (2 mM) of carbobenzoxyglycyl-L-methionine were reduced with sodium in liquid ammonia by the procedure of du Vigneaud *et al.* (4). 0.15 cc. of methyl iodide was then added and the ammonia was allowed to evaporate. The dry residue was dissolved in a few cc. of water, and the solution was neutralized with hydrobromic acid. After filtration, the solution was concentrated to dryness *in vacuo* and the residue was taken up in 22 cc. of hot 95 per cent ethanol. Upon cooling the solution, and the addition of a few drops of pyridine, crystals were obtained. The crude product was recrystallized from 70 per cent ethanol. Yield, 250 mg.; m.p., 196° (brown), 199.5° (decomposition); $[\alpha]_D^{22} = -10.0°$ (2 per cent in water). Hess and Sullivan (2) report a melting point of 140–145° for their preparation of this peptide.

$C_7H_{14}O_3N_2S$. Calculated. C 40.8, H 6.8, N 13.6
206.3   Found. " 41.1, " 6.7, " 13.5

Glycyl-D-methionine—This peptide was prepared in the same manner as the L isomer. M.p., 196° (brown), 200° (decomposition); $[\alpha]_D^{25} = +9.1°$ (1.2 per cent in water).

$C_7H_{14}O_3N_2S$. Calculated. 40.8, H 6.8, N 13.6
206.3   Found. " 40.7, " 6.9, " 13.5

Carbobenzoxyglycyl-L-methioninamide—5 gm. of the oily carbobenzoxy-glycyl-L-methionine methyl ester were prepared as described above, and dissolved in methanol previously saturated with dry ammonia at 0°. The solution was kept at room temperature for 53 hours, and then the excess ammonia and the solvent were removed *in vacuo*. The semicrystalline residue was recrystallized from ethyl acetate-petroleum ether and then from ethanol-water. Yield, 3.5 gm.; m.p., 131–134°.

$C_{16}H_{21}O_4N_3S$ (339.4). Calculated, N 12.4; found, N 12.3

Glycyl-L-methioninamide Acetate—1 gm. of carbobenzoxyglycyl-L-methioninamide was dissolved in a mixture of 15 cc. of methanol and 0.5 cc. of glacial acetic acid and was subjected to hydrogenolysis with palladium black as the catalyst. After 2 hours, the catalyst was removed by filtration and was washed with hot water. The combined filtrate and washings were concentrated *in vacuo* and the residue was dissolved in a small volume of methanol. Upon the addition of ethyl acetate, 0.6 gm. of the product was obtained. M.p., 108–110°. Recrystallization from ethyl acetate did not change the melting point.

$C_9H_{19}O_4N_3S$ (266.3). Calculated, N 15.8; found, N 15.8

Carbobenzoxy-L-methionylglycine Ethyl Ester—5 gm. (16.8 mM) of carbobenzoxy-L-methioninhydrazide were suspended in 40 cc. of water and dissolved by the addition of 5 cc. of glacial acetic acid and of 3 cc. of concentrated hydrochloric acid. The addition of 1.25 gm. of sodium nitrite (in 7 cc. of water) at 0° gave the semicrystalline azide which was extracted with ice-cold ether. The ether layer was washed at 0° with water, potassium bicarbonate solution, and water, dried over Na_2SO_4, and added to an ethereal solution of glycine ethyl ester (prepared from 10 gm. of the hydrochloride). The reaction mixture was kept at room temperature for 20 hours and then was washed with dilute hydrochloric acid, water, potassium bicarbonate solution, and water, and dried over Na_2SO_4. Removal of the solvent under reduced pressure gave a crystalline product which was recrystallized from ether-petroleum ether. Yield, 3.2 gm.; m.p., 93–95°.

$C_{17}H_{24}O_5N_2S$ (368.4). Calculated, N 7.6; found, N 7.7

Carbobenzoxy-D-methionylglycine Ethyl Ester—This compound was prepared in the same manner as the L isomer. M.p., 93–95°.

$C_{17}H_{24}O_5N_2S$ (368.4). Calculated, N 7.6; found, N 7.5

Carbobenzoxy-DL-methionylglycine Ethyl Ester—This compound was prepared in the same manner as the L isomer. M.p., 72–74°.

$C_{17}H_{24}O_5N_2S$ (368.4). Calculated, N 7.6; found, N 7.6

Carbobenzoxy-L-methionylglycine—2.06 gm. (5.6 mM) of the ester were dissolved in 35 cc. of methanol and 6.16 cc. of N NaOH (10 per cent excess) were added. The solution was kept at room temperature for 45 minutes, adjusted to pH 5 with N hydrochloric acid, and concentrated under reduced pressure. The resulting product was recrystallized from hot water. Yield, 1.65 gm.; m.p., 130–131°.

$C_{15}H_{20}O_5N_2S$ (340.4). Calculated, N 8.2; found, N 8.2

Carbobenzoxy-D-methionylglycine—This compound was prepared in the same manner as the L isomer. M.p., 129–130°.

$C_{15}H_{20}O_5N_2S$ (340.4). Calculated, N 8.2; found, 8.3

Carbobenzoxy-DL-methionylglycine—This compound was prepared in the same manner as the L isomer. M.p., 141–143°.

$C_{15}H_{20}O_5N_2S$ (340.4). Calculated, N 8.2; found, 8.1

L-Methionylglycine—500 mg. of carbobenzoxy-L-methionylglycine were dissolved in 15 cc. of methanol and subjected to hydrogenolysis in the presence of palladium black. After 3 hours, the catalyst was removed by filtration and washed on the filter with hot water. The combined filtrate and washings were concentrated under reduced pressure and the residue was recrystallized from water-ethanol. Yield, 200 mg.; m.p., 182–183° (decomposition); $[\alpha]_D^{21} = +86.8$ (2 per cent in water).

$C_7H_{14}O_3N_2S$. Calculated. C 40.8, H 6.8, N 13.6
206.3 Found. " 40.8, " 6.7, " 13.4

D-Methionylglycine—This compound was prepared in the same manner as the L isomer. M.p., 181° (decomposition); $[\alpha]_D^{23} = -88.0°$ (1.1 per cent in water).

$C_7H_{14}O_3N_2S$. Calculated. C 40.8, H 6.8, N 13.6
206.3 Found. " 40.7, " 6.9, " 13.5

DL-Methionylglycine—This compound was prepared in the same manner as the L isomer. M.p., 210° (decomposition).

$C_7H_{14}O_3N_2S$. Calculated. C 40.8, H 6.8, N 13.6
206.3 Found. " 41.0, " 7.0, " 13.6

Carbobenzoxy-L-methionylglycinamide—1.1 gm. (3 mM) of carbobenzoxy-L-methionylglycine ethyl ester were dissolved in 50 cc. of methanol previously saturated with dry ammonia at 0°. The solution was kept at room temperature for 2 days, and then the excess ammonia and the solvent were removed *in vacuo*, yielding an oily residue which was partitioned between water and ether. The ether layer was dried over Na_2SO_4 and

concentrated under reduced pressure. The residue was recrystallized from ethyl acetate-petroleum ether. Yield, 200 mg.; m.p., 120–122°.

$C_{15}H_{21}O_4N_3S$ (339.4). Calculated, N 12.4; found, N 12.5

Amide of 5-[2-(Methylthiol)ethyl]hydantoin-3-acetic Acid—The aqueous layer obtained in the preparation of carbobenzoxy-L-methionylglycinamide was concentrated to dryness *in vacuo*, and the residue was recrystallized from acetone-ether. Yield, 550 mg.; m.p., 138.5–139.5°.

$C_8H_{13}O_3N_3S$.   Calculated.   C 41.5, H 5.7, N 18.2
231.3   Found.   " 41.7, " 5.9, " 18.1

Amide of 5-Isobutylhydantoin-3-acetic Acid—5 gm. of carbobenzoxy-L-leucylglycine ethyl ester (19) were dissolved in 75 cc. of methanol previously saturated with dry ammonia at 0°. The solution was kept at room temperature for 4 days, and then was concentrated under reduced pressure. The resulting product was recrystallized from alcohol. Yield, 2.8 gm.; m.p., 183–184°.

$C_9H_{15}O_3N_3$.   Calculated.   C 50.7, H 7.1, N 19.7
213.2   Found.   " 50.8, " 7.0, " 19.7

Carbobenzoxy-α-L-glutamyl-L-methionine Methyl Ester—4 gm. of carbobenzoxy-L-glutamic acid anhydride (13) were added in small portions to an ethereal solution of L-methionine methyl ester (from 5.3 gm. of the hydrochloride). A reaction product began to separate before all of the anhydride had been added. The mixture was kept at room temperature for 3 hours, with intermittent shaking, and then the crystalline product was collected and dried (3 gm., Crop A). The filtrate was washed successively with water, dilute hydrochloric acid, and water, and dried over Na_2SO_4. The solvent was removed *in vacuo*, yielding an oil which soon crystallized (2.9 gm., Crop B). Crop B was recrystallized from ethyl acetate-petroleum ether and again from ethanol-water. M.p., 114–115°.

$C_{19}H_{26}O_7N_2S$ (426.5). Calculated, N 6.6; found, N 6.5

Carbobenzoxy-α-L-glutamyl-L-methionine—1.02 gm. (2.4 mM) of the methyl ester were saponified by shaking with 5 cc. of N NaOH for 15 minutes. The resulting solution was adjusted to pH 4 with N hydrochloric acid, yielding an oil which soon crystallized. Yield, 0.99 gm. After recrystallization from hot water, the product melted at 135–136°.

$C_{18}H_{24}O_7N_2S$ (412.5). Calculated, N 6.8; found, N 6.7

α-L-Glutamyl-L-methionine—400 mg. of the carbobenzoxy peptide were dissolved in 15 cc. of methanol and subjected to hydrogenolysis in the

presence of palladium black. Inactivation of the catalyst prevented completion of the reaction. After 6 hours, the catalyst was removed by filtration and washed on the filter with hot water. The combined filtrate and washings were concentrated *in vacuo* to a small volume. The solution was chilled, and the unchanged material (150 mg.) was removed by filtration. The filtrate was evaporated nearly to dryness, and the peptide was precipitated by the addition of absolute ethanol. Yield, 120 mg. After recrystallization from water-ethanol, the substance melted at 163–166°; $[\alpha]_D^{25} = + 18.8°$ (1.1 per cent in water).

$C_{10}H_{18}O_5N_2S$. Calculated. C 43.2, H 6.5, N 10.1
278.3   Found.   " 43.4, " 6.5, " 10.0

Carbobenzoxy-L-methionyl-L-methionine Methyl Ester—An ethereal solution of carbobenzoxy-L-methioninazide (from 5 gm. of the hydrazide) was added to an ethereal solution of L-methionine methyl ester (from 8 gm. of the hydrochloride). The reaction mixture was worked up as in the case of carbobenzoxy-L-methionylglycine ethyl ester. Yield, 4 gm. After recrystallization from ethyl acetate-petroleum ether, the substance melted at 98°.

$C_{19}H_{28}O_5N_2S_2$ (428.6). Calculated, N 6.5; found, N 6.5

Carbobenzoxy-L-methionyl-L-methionine—3 gm. of the ester were dissolved in 60 cc. of methanol and 7.7 cc. of N NaOH were added. After 30 minutes, the solution was acidified to pH 4 with N hydrochloric acid and the methanol was removed *in vacuo*. The product which separated was dissolved in ethyl acetate, from which it was extracted with an aqueous potassium bicarbonate solution. The bicarbonate solution was acidified, and the resulting crystalline material was recrystallized from ethyl acetate-petroleum ether. Yield, 1.7 gm.; m.p., 118–120°.

$C_{18}H_{26}O_5N_2S_2$ (414.5). Calculated, N 6.8; found, N 6.7

L-Methionyl-L-methionine—828 mg. of carbobenzoxy-L-methionyl-L-methionine were reduced with sodium in liquid ammonia. After a persistent blue color had been obtained, 0.3 cc. of methyl iodide were added, and the ammonia was allowed to evaporate. The dry residue was dissolved in 5 cc. of water, the solution was neutralized with hydrobromic acid, and then concentrated *in vacuo*. The addition of ethanol and ether gave a product which was recrystallized twice from 75 per cent ethanol. Yield, 220 mg.; m.p., 224–226.5° (decomposition); $[\alpha]_D^{25} = +26.5°$ (2 per cent in water).

$C_{10}H_{20}O_3N_2S_2$. Calculated. C 42.8, H 7.2, N 10.0
280.4   Found.   " 42.9, " 7.8, " 9.9

Carbobenzoxy-L-*methionyl*-L-*methioninamide*—1.0 gm. of carbobenzoxy-L-methionyl-L-methionine methyl ester was dissolved in 80 cc. of methanol previously saturated with dry ammonia at 0°. The solution was kept at room temperature for 68 hours and then was concentrated *in vacuo*. The solid residue was recrystallized from hot 90 per cent ethanol. Yield, 0.7 gm.; m.p., 196°.

$C_{18}H_{27}O_4N_3S_2$ (413.5). Calculated, N 10.2; found, N 10.2

Carbobenzoxy-L-*methionyl*-L-*tyrosine Ethyl Ester*—To an ethyl acetate solution of carbobenzoxy-L-methioninazide (from 2.97 gm. of the hydrazide) there was added an ethyl acetate solution of L-tyrosine ethyl ester (from 7.4 gm. of the hydrochloride). The mixture was worked up in the same manner as in the reaction of the azide with glycine ethyl ester. Yield, 2.5 gm.; m.p., 109–111°.

$C_{24}H_{30}O_6N_2S$ (474.6). Calculated, N 5.9; found, N 6.0

Carbobenzoxy-L-*methionyl*-L-*tyrosine*—1.1 gm. of the ester were dissolved in 25 cc. of methanol and 5.5 cc. of N NaOH were added. After 30 minutes, the reaction mixture was acidified to pH 4 with N hydrochloric acid, and the methanol was removed *in vacuo*. The oily product which separated was dissolved in ethyl acetate and extracted with an aqueous potassium bicarbonate solution. The bicarbonate solution was acidified, and the resulting crystalline product was recrystallized from ethanol-water. Yield, 0.67 gm.; m.p., 137–138.5°.

$C_{22}H_{26}O_6N_2S$ (446.5). Calculated, N 6.3; found, N 6.1

L-*Methionyl*-L-*tyrosine*—660 mg. of carbobenzoxy-L-methionyl-L-tyrosine were dissolved in 15 cc. of methanol and hydrogenated with palladium black as the catalyst. The peptide separated out during the reaction, and was dissolved by the addition of hot water. The catalyst was filtered off, and the filtrate was concentrated *in vacuo* to a small volume, whereupon the peptide crystallized. Ethanol was added to dissolve any unchanged material. Yield, 0.4 gm.; m.p., 260–266° (decomposition); $[\alpha]_D^{22} = +18.6°$ (2.1 per cent in 0.2 N hydrochloric acid).

$C_{14}H_{20}O_4N_2S$.   Calculated.   C 53.8, H 6.5, N 9.0
312.4   Found.   " 54.0, " 6.5, " 8.8

Carbobenzoxy-L-*methionylglycylglycine Ethyl Ester*—To an ethereal solution of carbobenzoxy-L-methioninazide (from 6 gm. of the hydrazide) there was added an ethyl acetate solution of glycylglycine ethyl ester (from 10 gm. of the hydrochloride). Crystallization of the coupling product began at once. After 20 hours, the reaction mixture was filtered, and the filtrate was worked up as in the case of carbobenzoxy-L-methionyl-

glycine ethyl ester. The total yield was 6.5 gm. After recrystallization from ethyl acetate-petroleum ether, the substance melted at 131–133°.

$C_{19}H_{34}O_6N_2S$ (422.5). Calculated, N 9.9; found, N 9.8

Carbobenzoxy-L-methionylglycylglycine—2.5 gm. of the ester were dissolved in 50 cc. of warm methanol, and 6 cc. of N NaOH were added. After 30 minutes, the solution was acidified to pH 4 with N hydrochloric acid and the methanol was removed under reduced pressure. The crystals which separated upon chilling the solution were recrystallized from hot water. Yield, 2.0 gm.; m.p., 137–138°.

$C_{17}H_{20}O_6N_3S$ (394.4). Calculated, N 10.6; found, N 10.4

L-*Methionylglycylglycine*—800 mg. of carbobenzoxy-L-methionylglycyl-glycine were dissolved in a mixture of 17 cc. of methanol and 0.5 cc. of glacial acetic acid and hydrogenated with palladium black as the catalyst. Inactivation of the catalyst prevented completion of the reaction. After 6 hours, the catalyst was removed by filtration and washed with methanol and hot water. The filtrate and washings were concentrated to about 1 cc. *in vacuo*, and the peptide was precipitated by the addition of 10 cc. of 95 per cent ethanol. Yield, 375 mg. After two recrystallizations from water-ethanol, the substance melted at 208–209° (decomposition), and had a rotation of $[\alpha]_D^{23} = +73.1°$ (1.9 per cent in water).

$C_9H_{17}O_4N_3S$.   Calculated.   C 41.1, H 6.5, N 16.0
263.3   Found.   " 41.3, " 6.6, " 14.9

Carbobenzoxy-L-threonine Methyl Ester—An ethyl acetate solution of L-threonine methyl ester (from 2 gm. of L-threonine) was treated with 3.5 cc. of carbobenzoxy chloride in the presence of an aqueous potassium bicarbonate solution. The excess chloride was destroyed with pyridine and the ethyl acetate layer was washed successively with water, dilute hydrochloric acid, and water, and dried over Na_2SO_4. The ethyl acetate solution was concentrated *in vacuo*, and the resulting product was recrystallized from ethyl acetate-petroleum ether. Yield, 3.0 gm.; m.p., 89–91°.

$C_{13}H_{17}O_4N$ (267.3). Calculated, N 5.2; found, N 5.1

Carbobenzoxy-L-threoninamide—2.9 gm. of the ester were dissolved in 50 cc. of methanol previously saturated with dry ammonia at 0°. The solution was kept at room temperature for 48 hours, and then was concentrated *in vacuo*, yielding an oil which was crystallized from ethyl acetate-petroleum ether. Yield, 2.5 gm. This crude product was dissolved in ethyl acetate and the solution was washed with dilute hydrochloric acid and water, and dried over Na_2SO_4. Upon evaporation of the solvent

in vacuo, there was obtained a crystalline product which was recrystallized from ethyl acetate-petroleum ether. In the melting point tube, the compound decomposed on slow elevation of the temperature, but if the bath were preheated to 70° before insertion of the tube, and the temperature raised rapidly, the substance melted sharply at 82–83°.

<div align="center">$C_{12}H_{16}O_4N_2$ (252.3). Calculated, N 11.1; found, N 10.9</div>

The authors wish to acknowledge with thanks the valuable technical assistance of Miss P. Elizabeth Driscoll and of Miss Mary E. Mycek.

SUMMARY

1. The following peptides of methionine have been synthesized: glycyl-L- and D-methionine, L- and D-methionylglycine, α-L-glutamyl-L-methionine, L-methionyl-L-methionine, L-methionyl-L-tyrosine, and L-methionyl-glycylglycine. In addition, there have been prepared a number of peptide derivatives containing L- and D-methionine residues.

2. Carbobenzoxy-L-methioninamide has been found to be a sensitive substrate for papain and ficin, as judged by a comparative study of the action of these enzymes on an extensive series of acylamino acid amides.

3. The action of crystalline carboxypeptidase on acylated dipeptides containing L-methionine residues has been investigated, and the results are in general agreement with earlier conclusions concerning the influence of structural changes in substrates for this enzyme on their relative rates of hydrolysis.

4. Crystalline pepsin hydrolyzes carbobenzoxy-L-methionyl-L-tyrosine and L-methionyl-L-tyrosine at pH 4.

5. The action of aqueous extracts of desiccated swine kidney cortex on several peptides of L-methionine has been examined, and, as in the case of the hydrolysis of L-leucyl peptides, the enzymatic hydrolysis of L-methionyl peptides was found to be markedly activated by manganese ions.

BIBLIOGRAPHY

1. Dekker, C. A., and Fruton, J. S., *J. Biol. Chem.*, **173**, 471 (1948).
2. Hess, W. C., and Sullivan, M. X., *J. Am. Chem. Soc.*, **63**, 881 (1941).
3. Fruton, J. S., *Advances in Protein Chem.*, **5**, in press (1949).
4. du Vigneaud, V., Audrieth, L. F., and Loring, H. S., *J. Am. Chem. Soc.*, **52**, 4500 (1930).
5. Stekol, J. A., *J. Biol. Chem.*, **140**, 827 (1941).
6. du Vigneaud, V., Dyer, H. M., and Harmon, J., *J. Biol. Chem.*, **101**, 719 (1933).
7. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **145**, 253 (1942).
8. Wessely, F., Kemm, E., and Mayer, J., *Z. physiol. Chem.*, **180**, 64 (1929).
9. Gränacher, C., and Landolt, H., *Helv. chim. acta*, **10**, 799 (1927).

10. Fischer, E., *Ber. chem. Ges.*, **38**, 605 (1905).
11. Behrens, O. K., and Bergmann, M., *J. Biol. Chem.*, **129**, 587 (1939).
12. Fruton, J. S., *J. Biol. Chem.*, **146**, 463 (1942).
13. Bergmann, M., and Zervas, L., *Ber. chem. Ges.*, **65**, 1192 (1932).
14. Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, **127**, 643 (1939).
15. Grassmann, W., *Biochem. Z.*, **279**, 131 (1935).
16. Kaufman, S., Neurath, H., and Schwert, G. W., *J. Biol. Chem.*, **177**, 793 (1949).
17. Fruton, J. S., in Green, D. E., Currents in biochemical research, New York, 123 (1946).
18. Bergmann, M., and Fruton, J. S., *Advances in Enzymol.*, **1**, 63 (1941).
19. Bergmann, M., Zervas, L., and Fruton, J. S., *J. Biol. Chem.*, **111**, 225 (1935).
20. Bergmann, M., Zervas, L., and Fruton, J. S., *J. Biol. Chem.*, **115**, 593 (1936).
21. Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **138**, 231 (1941).
22. Rocha e Silva, M., and Andrade, S. O., *J. Biol. Chem.*, **149**, 9 (1943).
23. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **145**, 247 (1942).
24. Stahmann, M. A., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **164**, 753 (1946).
25. Smith, E. L., *J. Biol. Chem.*, **175**, 39 (1948).
26. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **134**, 225 (1940).
27. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **118**, 405 (1937).
28. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **127**, 627 (1939).
29. Harington, C. R., and Pitt Rivers, R. V., *Biochem. J.*, **38**, 417 (1944).
30. Schott, H. F., Larkin, J. B., Rockland, L. B., and Dunn, M. S., *J. Org. Chem.*, **12**, 490 (1947).
31. Simmonds, S., Tatum, E. L., and Fruton, J. S., *J. Biol. Chem.*, **169**, 91 (1947).
32. Smith, E. L., and Bergmann, M., *J. Biol. Chem.*, **138**, 789 (1941).

A METHOD FOR THE COLORIMETRIC DETERMINATION OF CALCIUM AND MAGNESIUM IN SMALL AMOUNTS OF URINE, STOOL, AND FOOD*

By GEORGE D. MICHAELS, CARL T. ANDERSON, SHELDON MARGEN, AND LAURANCE W. KINSELL

(*From the Division of Medicine, University of California Medical School, and the Metabolic Research Unit, United States Naval Hospital-University of California Medical School, Oakland and San Francisco*)

This report presents a method for the quantitative determination of magnesium and calcium in a single specimen of urine, stool, and food by formation and colorimetric determination of their respective phosphates. The method makes use of some of the principles advanced by Roe and Kahn (1) for determination of serum calcium, and by Denis (2) for serum magnesium. Phosphorus determinations are performed by the method of Fiske and Subbarow (3).

Principle

The principle involved relates to the conversion of magnesium and calcium to their phosphates; determination of the total phosphate (Mg + Ca); precipitation of the calcium as oxalate, and of the magnesium (after separation) as magnesium ammonium phosphate. The calcium is then redissolved and reprecipitated as calcium phosphate. Both phosphates are then determined, and should equal the total phosphate above noted.

Reagents—

1. Phosphorus reagents (Fiske and Subbarow (3)).
2. 5 per cent ammonium phosphate (secondary) solution.
3. Ammonium hydroxide, c.p., 28 per cent.
4. 20 per cent sodium acetate solution.
5. 2.5 per cent oxalic acid.
6. Alcohol wash containing 25 ml. of 5 per cent ammonium chloride, 100 ml. of ammonium hydroxide, 200 ml. of 95 per cent ethyl alcohol, 15 ml. of amyl alcohol, and 160 ml. of distilled water.
7. 1:4 dilution of concentrated hydrochloric acid.
8. 1:50 dilution of 28 per cent ammonium hydroxide.
9. Indicator (methyl orange, pH range 3.1 to 4.4).

Method

Calcium and Magnesium Analysis of Urine

1. To a 15 ml. conical centrifuge tube add 10 ml. of well shaken urine, 2 drops of methyl orange, concentrated hydrochloric acid dropwise, to a red color, and 1 ml. of 5 per cent ammonium phosphate slowly with shaking.

2. Add 2 ml. of ammonium hydroxide (28 per cent), mix, and let stand for at least 1 hour.

3. Centrifuge, decant, and wash the precipitate three times with 5 ml. of alcohol wash.

4. Dissolve the precipitate with 0.5 ml. of 1:4 hydrochloric acid and wash quantitatively into a 10 ml. volumetric flask, bring to volume with distilled water, and mix.

5. 1 or 2 ml. of this are then used for total phosphate determination.

6. 5 ml. of the aliquot from item (4) are taken and 1 ml. of 2.5 per cent oxalic acid is added, as well as a drop of methyl orange. Sodium acetate solution is then added slowly until the pH is approximately 4.0; *i.e.*, until the indicator just turns from red to orange. The mixture is then allowed to stand for 4 hours or more for the complete precipitation of the calcium oxalate. This assures complete calcium precipitation and magnesium solution (pH below 5.0 and above 2.5), as shown by Washburn and Shear (4).

7. The precipitate of calcium oxalate is then centrifuged and washed twice with 3 ml. of 2 per cent ammonium hydroxide. The supernatant and the washings are saved for the magnesium determination.

8. The calcium oxalate precipitate is dissolved in 0.5 ml. of 1:4 hydrochloric acid and 5 ml. of water. 0.5 ml. of 5 per cent ammonium phosphate and 2 ml. of ammonium hydroxide are added and mixed to reprecipitate the calcium as the phosphate. After allowing this to stand for 1 hour or more, it is centrifuged and the precipitate washed twice with the alcohol wash. (The 40 per cent alcohol wash will *not* precipitate phosphates of sodium, potassium, and ammonium.) The precipitate is then dissolved with 0.5 ml. of 1:4 hydrochloric acid and the entire amount or a portion may be used for the phosphorus determination, depending upon the amount of calcium present.

9. To the supernatant and the washing from the above, 0.5 ml. of ammonium phosphate solution and 2.0 ml. of strong ammonia are added and allowed to stand for 1 hour or longer. This precipitate (magnesium ammonium phosphate) is centrifuged and washed twice with 5 ml. of alcohol wash and then redissolved in 1:4 hydrochloric acid. Phosphate analysis is then performed upon (1) the total phosphate from step (5),

(2) the calcium phosphate from step (8), (3) the magnesium ammonium phosphate from step (9).

Actual phosphate determinations are performed by the method of Fiske and Subbarow, as previously noted. Colorimetry is performed with any

TABLE I

Calcium-Phosphorus and Magnesium-Phosphorus Ratios in Aqueous Solutions Containing Known Amounts of Calcium

Specimen No.	Ca added	P recovered	Ca:P	Mg added	P recovered	Mg:P
	mg.	*mg.*		*mg.*	*mg.*	
1	0.273	0.167	1.63	0.512	0.654	0.783
2	0.273	0.168	1.62	0.512	0.654	0.783
3	0.546	0.336	1.62	1.025	1.293	0.792
4	0.546	0.336	1.62	1.025	1.308	0.783
5	0.819	0.501	1.63	1.536	1.982	0.775
6	0.819	0.504	1.62	1.536	1.962	0.783
7				3.075	3.914	0.785
8				3.075	3.974	0.774
Average.............		1.62				0.782

TABLE II

Predicted and Actual Phosphorus Recovery in Aqueous Solutions, Containing Known Amounts of Calcium and Magnesium

With the factors noted in Table I, excellent correspondence is obtained between predicted and actual values.

Specimen No.	Ca added	Mg added	Calculated P (Ca × 1.62 + Mg × 0.782)	Actual P recovered	Per cent recovery
	mg.	*mg.*		*mg.*	
1	0.273	0.512	0.821	0.820	100.0
2	0.273	0.512	0.821	0.815	99.3
3	0.546	1.025	1.642	1.631	99.3
4	0.546	1.025	1.642	1.639	99.8
5	0.819	1.536	2.463	2.453	99.6
6	0.819	1.536	2.463	2.458	99.8

standard photoelectric colorimeter, having a green filter with an approximate spectral range of 500 to 570 mμ.

Calcium and Magnesium Analysis of Food and Stool

Stool and food are dry-ashed for a minimum of 12 hours at a temperature of 500–600°.

The amount of ash derived from 2 gm. of dry stool or food is dissolved in 5 ml. of warm 1:4 hydrochloric acid. This solution is made up to 50 ml. with distilled water in a volumetric flask. The procedure thereafter is as described for urine, except that the size of the aliquot will vary with the calcium and magnesium concentration. Best results are obtained with a final phosphorus concentration of 0.3 to 0.8 mg. per 100 ml.

Calculation (See Below for Derivation)—Mg. of calcium = mg. of phosphorus × factor 1.62; mg. of magnesium = mg. of phosphorus × factor 0.782.

Analysis of Known Calcium-Magnesium Solutions

Since silicates are known to interfere with the phosphorus determination, we have avoided the use of strong bases in the conversion of calcium

TABLE III

Recovery of Added Calcium and Magnesium from Pooled Urine, by Use of Ca:P and Mg:P Factors from Table I

	Ca added	Mg added	Ca recovered	Mg recovered	Recovery		Total P calculated	Total P recovered	Recovery Actual P
					Ca	Mg			Calculated P
	mg.	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*mg.*	*mg.*	*per cent*
Urine			1.293	0.485				1.408	
" + Ca	0 546		1.849	0.480	100.6	98.9	1.744	1.745	100.0
" + Mg		0.512	1.293	0.992	100.0	99.4	2.062	2.081	100.9
" + Ca + Mg	0.546	0.512	1.839	0.998	100.0	100.0	2.398	2.400	100.0
" + " + "	0.819	1.025	2.132	1.500	100.9	99.3	3.217	3.249	100.9
" + " + "	0.546	1.025	1.819	1.518	98.9	100.5	3.052	3.079	100.8

and magnesium to their phosphates. Ammonium hydroxide, being a weak base, does not form silicates and does form a compound or compounds having a constant calcium-phosphorus ratio of 1.62:1, as shown in Table I. The magnesium to phosphorus ratio is 0.782:1.[1] This would indicate the formation of magnesium ammonium phosphate, whereas, in the case of calcium, apparently an equal mixture of tricalcium phosphate and ammonium calcium phosphate results. Analysis of known mixtures of calcium and magnesium in distilled water gave rise to the above ratios. Recovery of total phosphates ranged from 100 to 99.3 per cent, as shown in Table II.

A second experiment similar to the one just described was performed upon previously analyzed pooled urine to which were added known quantities of calcium and magnesium. The data are recorded on Table III. Calcium and magnesium are recovered quantitatively.

[1] The magnesium standard was prepared as described by Jones (5).

Table IV shows the comparisons of the calcium values on food, urine, and stools by McCrudden's method (6) and the one here described.

Considerable variation is noted in some instances. It is worthy of emphasis that, as shown in Table IV, values obtained by the method herein described are reproducible.

To date 209 urinalyses, thirty-two diet analyses, and forty-nine stool analyses have been performed in duplicate in this laboratory by the method reported. Low variability is a consistent finding.

TABLE IV

Calcium Determination in Food, Stool, and Urine; Comparison of Two Methods

Specimens analyzed		McCrudden's method, duplicate analyses		Variation per sample	Phosphate method, duplicate analyses		Variation per sample
		gm. per 100 ml.	*gm. per 100 ml.*	*mg.*	*gm. per 100 ml.*	*gm. per 100 ml.*	*mg.*
Food	Amigen	0.458	0.440	18	0.444	0.440	4
	Milk 1	0.114	0.112	2	0.102	0.102	0
	" 2	0.118	0.115	3	0.117	0.116	1
	Blended food	0.019	0.019	0	0.021	0.021	0
	Veal	0.017	0.018	1	0.011	0.011	0
	Beef	0.008	0.007	1	0.009	0.009	0
		gm. per 24 hrs.	*gm. per 24 hrs.*		*gm. per 24 hrs.*	*gm. per 24 hrs.*	
Urine	Sm	0.102	0.100	2	0.098	0.097	1
	Pa	0.123	0.122	1	0.125	0.124	1
	Ma$_1$	0.074	0.072	2	0.082	0.082	0
	Ma$_2$	0.073	0.072	1	0.072	0.072	0
Stool	Sm	0.651	0.623	28	0.676	0.673	3
	Pa	1.866	1.834	32	1.854	1.854	0
	Ma$_1$	1.410	1.410	0	1.370	1.370	0
	Ma$_2$	1.120	1.034	86	1.010	1.002	8

SUMMARY

1. The determination of calcium phosphate, magnesium phosphate, and combined phosphate, as described, provides a constant check of the accuracy of the procedure in terms of loss of material; it does *not* provide a check on the magnesium-calcium separation. Therefore, proper control of pH is essential, as noted above.

2. Initial precipitation of calcium and magnesium in the presence of an excess of phosphate, and in the absence of strong alkali eliminates interfering substances such as sulfates and silicates, as suggested by Fiske and Logan (7) and Roe and Kahn (1).

3. The simplicity and accuracy of the method commend its use, particularly when multiple urine and stool determinations or small amounts of material are required, as in the case of metabolic studies generally.

BIBLIOGRAPHY

1. Roe, J. H., and Kahn, B. S., *J. Biol. Chem.*, **67**, 585 (1926); **81**, 1 (1929)
2. Denis, W., *J. Biol. Chem.*, **52**, 411 (1922).
3. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
4 Washburn, M. L., and Shear, M. J., *J. Biol. Chem.*, **99**, 21 (1931–32).
5. Jones, W., *J. Biol. Chem.*, **25**, 87 (1916).
6. McCrudden, F. H , *J. Biol. Chem.*, **10**, 187 (1911–12).
7 Fiske, C. H., and Logan, M. A., *J Biol. Chem.*, **93**, 211 (1931).

THE EFFECT OF METHANOL ON THE HYDROLYSIS OF ACETYL-L-TYROSINAMIDE BY CHYMOTRYPSIN*

BY SEYMOUR KAUFMAN† AND HANS NEURATH

(From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina)

In recent studies on the kinetics and specificity of chymotrypsin several new peptide and ester substrates were introduced (2–5). Some of these were relatively insoluble in aqueous salt solutions but sufficiently soluble in methanol-water mixtures to enable the determination of enzymatic hydrolysis in the presence of 20 or 30 per cent methanol. Measurements of the rate of hydrolysis of two representative substrates, *i.e.* glycyl-L-tyrosinamide (GTA) and benzoyl-L-tyrosine ethyl ester (BTEE), have shown that at constant initial substrate concentration the apparent proteolytic coefficient,[1] C, decreased logarithmically with increasing methanol concentration, up to about 35 per cent methanol (3).

As an extension of these observations, the effect of methanol on both steps of the postulated two-step reaction mechanism of enzymatic reactions (3, 7, 8) has now been determined. These two rate-contributing steps, previously considered, are (1) the reversible formation of the enzyme-substrate complex, (ES), and (2) its conversion into the activated complex, $(ES)^*$. While additional, intermediate steps may exist,[2] their consideration does not seem to contribute profitably to the interpretation of the present analyses.

The substrate chosen for this investigation was acetyl-L-tyrosinamide (ATA) which is hydrolyzed faster than GTA; the even more susceptible substrate benzoyl-L-tyrosinamide (BTA) is appreciably soluble only in 30 per cent methanol.

EXPERIMENTAL

Substrate—Acetyl-L-tyrosinamide was synthesized from acetyl-L-tyrosine ethyl ester (3, 9) by dissolving the ester in absolute methanol and saturating it with dry ammonia gas at 0°. The clear solution was allowed to

---

* A preliminary account of this work was included in a recent publication (1).

† Postdoctoral Fellow of the United States Public Health Service, National Institutes of Health.

[1] Defined as the first order reaction constant, calculated from decimal logarithms, per mg. of enzyme N per cc., at constant initial substrate concentration (6).

[2] Bull, H. B., and Currie, B. T., to be published. Private communication from Dr. H. B. Bull.

stand at room temperature for 2 days and then concentrated under reduced pressure. White crystals were obtained. M.p. 222–224°.

<div align="center">Calculated, N 12.6; found, N 12.4</div>

Enzyme—The preparation of chymotrypsin was the same as that previously described (2, 3).

Methods—All experiments were performed at 25° in a solution containing phosphate buffer of pH 7.8 which, if necessary, was fortified with LiCl to a total ionic strength of 0.292. Because of the limited solubility of the buffer salts in aqueous methanol, the total phosphate concentration was decreased within the range of 0.1 M to 0.079 M as the methanol concentration was increased from 0 to 20.8 volumes per cent. The proper amounts of LiCl were added to maintain the ionic strength at the constant level. Since the substrate was only difficultly soluble in aqueous solutions, supersaturated solutions were prepared which remained stable for more than 6 hours. Blank determinations for each experiment indicated that none of the amide had been hydrolyzed under these conditions. A weighed amount of substrate was heated in buffer solution until it was completely dissolved, and after cooling, made up to volume with buffer. The calculated amount of absolute methanol was then added, followed by the addition of a known, small volume of a freshly prepared solution of chymotrypsin.

Rates of hydrolysis were determined by measuring the liberated ammonia with the modified Conway method (2). Enzyme concentrations were adjusted so as to yield about 70 per cent hydrolysis in about 80 to 90 minutes. Nitrogen analyses were carried out with the semimicro-Kjeldahl method.

<div align="center">*Results*</div>

In accordance with previous analyses (3, 8), the present data were evaluated in terms of K_m and k', where K_m is the equilibrium constant for the reversible formation and disappearance of the stable enzyme-substrate complex, (ES), and k' the specific rate constant for the formation of the activated complex, $(ES)^*$, from (ES). K_m is measured in moles of substrate per liter, and k' in moles of substrate per liter hydrolyzed per minute per mg. of enzyme N per cc.[3] These constants were obtained from a plot of the equation (10)

$$\frac{1}{v} = \frac{K_m}{V_{max.}} \frac{1}{a} + \frac{1}{V_{max.}} \tag{1}$$

---

[3] k'_0 is the specific rate constant, in sec.$^{-1}$, and is related (3) to k' by $k'_0 = k' \times$ (molecular weight of chymotrypsin)/(60 × 6.25).

where a is the initial substrate concentration in moles per liter, v the initial velocity expressed as moles per liter hydrolyzed during the initial 5 minute period, and V_{max} the maximum velocity prevailing when all of the enzyme is tied up in the enzyme-substrate complex. Accordingly, $V_{max}./5 = k'e$, where e is mg. of enzyme N per cc. Initial velocities were determined by extrapolation from the linear first order reaction plots to
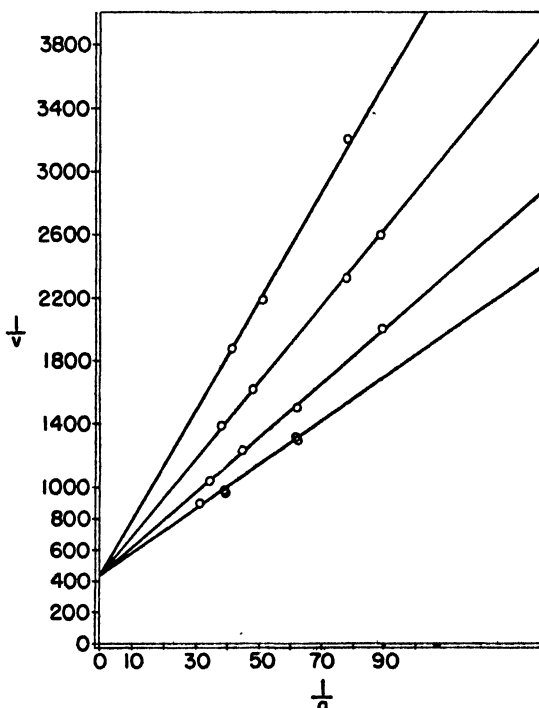
FIG. 1. A plot of equation (1) for the determination of K_m and k' for the chymotryptic hydrolysis of ATA in aqueous methanol solutions. The straight lines, in order of increasing slopes, refer to solutions containing 0, 8.33, 15.0, and 20.8 volumes per cent of methanol, respectively. For detailed conditions of the experiments, see the text.

which all measurements adhered at constant initial substrate concentration.

At constant solvent composition and constant enzyme concentration, measurements in four different substrate concentrations, ranging from about 0.01 to 0.03 M, were performed to determine the slopes and intercepts of the straight lines which result from a plot of equation (1). These results are shown in Fig. 1 which demonstrates that the ordinate intercept is practically independent of solvent composition, whereas the slopes

increase as methanol concentration is increased. $V_{max.}$ and k' were evaluated by plotting a rearranged form of equation (1), *i.e.* a/v *versus* a. The results are given in Table I in which the calculated values for $C_{max.}$,

<div align="center">TABLE I</div>

Hydrolysis of Acetyl-L-tyrosinamide by Chymotrypsin in Relation to Methanol Concentration

Methanol		Enzyme	K_m	k'	$C_{max.}$*
vol per cent	*M*	*mg N per cc.*	10^{-3} *M*	10^{-2}	
0	0	0.141	3.26	2.72	3.9
8.33	2.08	0.140	4.06	3.16	3.1
15.0	3.74	0.155	5.69	2.89	2.2
20.8	5.17	0.167	8.06	2.89	1.6
Average....				2.90 ±0.12	

* Defined by equation (2).

the maximum proteolytic coefficient (8), are also included. These were calculated from the relation (8)

$$\frac{k'}{2.3K_m} \tag{2}$$

<div align="center">DISCUSSION</div>

The data shown in Fig. 1 and Table I demonstrate clearly that, in the present enzyme-substrate system, the addition of methanol to the solvent does not affect k', the rate constant for the formation of the activated complex $(ES)^*$ from its stable precursor. This finding suggests that the change in the activity of water by the addition of methanol is not a rate-limiting factor.

Although the plots shown in Fig. 1 resemble those characteristic of competitive inhibition of enzymatic reactions (10, 11), the phenomenon described herein is of a different nature. Visual inspection of the relative slopes and actual calculations reveal that the apparent enzyme inhibitor dissociation constant, K_i, considering methanol as the inhibitor, decreases markedly as methanol concentration increases, instead of remaining independent of the latter.

A more likely explanation of the present findings is that methanol, by virtue of its lowering effect on the dielectric constant of the solvent, affects the rates of formation, k_1, and of dissociation, k_2, of the complex (ES), as represented by equation (3). The calculated dielectric constants

(12) vary from about 78.5 for water to 70.6 for the highest methanol concentration which was investigated (20.8 volumes per cent, *i.e.* 5.17 M).

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} (ES) \overset{k'}{\longrightarrow} (ES)^* \cdots \tag{3}$$
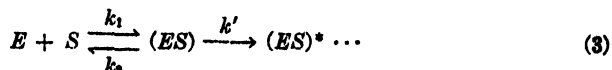
K_m, the enzyme-substrate equilibrium constant, is related to the rate constants (8), k_1, k_2, and k', by

$$K_m = \frac{k_2 + k'}{k_1} \tag{4}$$

Since in the above equation k' remains constant, no direct or reciprocal proportionality between K_m and methanol concentration is to be ex
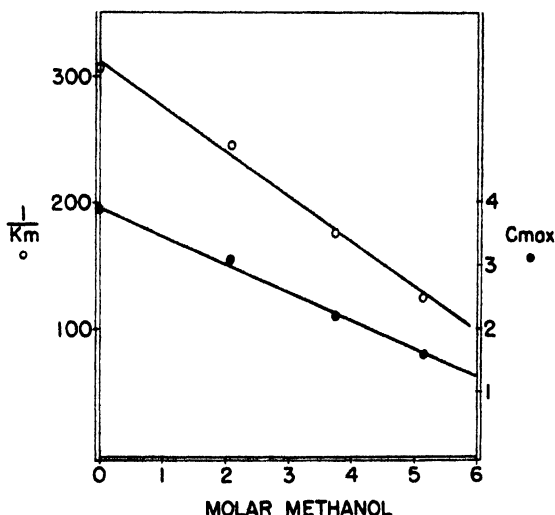
Fig. 2. A plot showing the linear relations between $1/K_m$ (O) and C_{max} (●), respectively, and methanol concentration (moles per liter). For a definition of K_m and C_{max}, see equations (1) and (2) and the text.

pected. However, inspection of the data reveals $1/K_m$ to be a linear function of methanol concentration, as shown in the upper plot of Fig. 2. This linearity can only obtain if k_2 is likewise independent of methanol concentration or if this quantity is negligibly small as compared to k' (13). The latter of these alternative interpretations is more realistic, since it is unlikely that a change in dielectric constant has an unidirectional influence on an equilibrium. It follows from these interpretations that, in the present system, $K_m = k'/k_1$; *i.e.*, the enzyme-substrate equilibrium constant is merely dependent on the relative rates of formation

and disappearance of (ES). It also follows that k_1 decreases with decreasing dielectric constant of the solvent, and that the maximum proteolytic coefficient, $C_{max.}$, defined by equation (2), is simply k_1. Accordingly, $C_{max.}$ should be directly proportional to methanol concentration, and it actually is, as shown in the lower plot of Fig. 2.[4] In accordance with expectations (8), it was also found that the concentration dependence of first order reaction constants at constant solvent composition decreases as methanol concentration is increased.[5]

The linear decrease of k_1 with decreasing dielectric constant of the medium may offer some clue to the nature of the forces involved in specific enzyme-substrate interaction. The rates of interaction between oppositely charged ions, or between ions and neutral molecules, generally increase as the dielectric constant of the medium is lowered. It is only for interaction between ions of like sign of charge, or between dipolar molecules forming a polar product, that the rates decrease as the dielectric constant decreases (14). A decision between the latter alternative has to await studies on the influence of ionic strength on reaction rates of the present system, which should yield qualitatively different results for interaction between ions of like sign of charge and for the interaction between dipoles (14). However, since the present substrate is an uncharged dipole, specific interaction with a dipolar grouping of the active centers on the enzyme surface could lead to an ionized product if protons or hydroxyl ions are simultaneously taken up from the solvent. Experiments to be published elsewhere[6] indeed suggest that chymotryptic hydrolysis of specific peptide and ester substrates involves the transfer of hydroxyl ions from solution to the enzyme-substrate complex.

[4] Provided that the linear relations shown in Fig. 2 also apply to higher concentrations of methanol, these lines extrapolate to $K_m \rightarrow \infty$ or $k_1 \rightarrow 0$ at about 8.9 M methanol, equivalent to a dielectric constant of about $D = 64.5$. The significance of this extrapolation remains to be elucidated.

[5] It was previously reported (3) that at constant initial substrate concentration, $\log C$ for the chymotryptic hydrolysis of GTA and of BTEE was a linear function of methanol concentration. Although at an initial concentration of 0.033 M ATA, $\log C$ was also linearly related to methanol concentration, a linear relation of C fitted the data equally well, in contrast to similar plots for GTA and BTEE, which were distinctly curvilinear. The present interpretation of $k_1 \ll k'$ may, therefore, be characteristic for the specific substrate, ATA, but not generally applicable to all substrates for a given enzyme.

[6] Snoke, J. E., and Neurath, H., manuscript in preparation.

SUMMARY

The effect of methanol on the chymotryptic hydrolysis of acetyl-L-tyrosinamide was investigated and analyzed in terms of the two-step reaction mechanism previously described. It was found that the rate of activation of the stable enzyme-substrate complex was independent of methanol concentration, within the range of 0 to 20.8 volumes per cent of methanol. In contrast, the enzyme-substrate equilibrium constant, K_m, increased with increasing methanol concentration.

Analysis of the kinetic data has led to the conclusion that in the present system the rate of dissociation of the enzyme-substrate complex into reactants is negligibly small as compared to the rate of activation, and that methanol, by virtue of its lowering effect on the dielectric constant of the medium, lowers the rate of combination of free enzyme and substrate. Tentative conclusion on the nature of the forces involved in enzyme-substrate combination have been formulated.

BIBLIOGRAPHY

1. Kaufman, S., and Neurath, H., *Arch. Biochem.*, **21,** 245 (1949).
2. Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J. E., *J. Biol. Chem.*, **172,** 221 (1948).
3. Kaufman, S., Neurath, H., and Schwert, G. W., *J. Biol. Chem.*, **177,** 793 (1949).
4. Snoke, J. E., and Neurath, H., *Arch. Biochem.*, **21,** 351 (1949).
5. Kaufman, S., and Neurath, H., *Arch. Biochem.*, **21,** 437 (1949).
6. Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **138,** 231 (1941).
7. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49,** 333 (1913).
8. Elkins-Kaufman, E., and Neurath, H., *J. Biol. Chem.*, **175,** 893 (1948).
9. du Vigneaud, V., and Meyer, C. E., *J. Biol. Chem.*, **98,** 295 (1932).
10. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658 (1934).
11. Ebersole, E. R., Guttentag, C., and Wilson, P. W., *Arch. Biochem.*, **3,** 399 (1943–44).
12. Åkerlöf, G., *J. Am. Chem. Soc.*, **54,** 425 (1932).
13. Van Slyke, D. D., and Cullen, G. E., *J. Biol. Chem.*, **19,** 141 (1914).
14. Glasstone, S., Laidler, K. J., and Eyring, H., The theory of rate processes, New York and London (1941).

STUDIES ON THE EFFECT OF THYROID AND METHYL-THIOURACIL ON THE GLUCOSE TOLERANCE TEST IN NORMAL AND SCORBUTIC GUINEA PIGS

By SACHCHIDANANDA BANERJEE and NARESH CHANDRA GHOSH

(*From the Department of Physiology, Presidency College, Calcutta, India*)

Patients with hyperthyroidism often have high fasting blood sugar levels and glucose tolerance curves of a diabetic·type. These become normal after thyroidectomy (1–7). The glycogen content of the liver of rats is decreased when experimental hyperthyroidism is produced (8). Prina (9) has shown that intraperitoneal injection of thiouracil causes a definite increase in the glycogen content of the liver of guinea pigs. Sure and Theis (10) have reported that if a toxic dose of thyroxine is fed to rats the vitamin C content of the adrenal, thymus, and kidney is greatly reduced. Demole and Ippen have observed (11) that the loss in weight of guinea pigs after injection of thyroxine can be prevented by increasing the dose of vitamin C. A large dose of ascorbic acid promotes the general vitality and improves the appearance of guinea pigs on a thyroid diet (12). Monetti (13) has observed that administration of a non-toxic dose of thyroxine enhances the course of scurvy in animals on the same diet without the hormone. Lewis (14) has reported that in thyrotoxic patients the excretion of vitamin C is below normal and becomes normal after thyroidectomy. It has been observed by us (15) that scorbutic guinea pigs show a diminished glucose tolerance, diminished glycogen content of the liver, and diminished insulin content of the pancreas. It was therefore of interest to ascertain whether or not the metabolism of the thyroid gland is also interfered with during the development of scurvy. In the present paper studies of the effects of feeding desiccated thyroid gland and methylthiouracil on the glucose tolerance of both normal and scorbutic guinea pigs are presented.

EXPERIMENTAL

Healthy, male, growing guinea pigs, varying between 300 and 380 gm. in weight, were fed six different diets for a period of 3 weeks. The diets were (1) the scorbutic diet only (16); (2) the scorbutic diet containing 1 per cent methylthiouracil; (3) the scorbutic diet with 0.05 per cent desiccated thyroid;[1] (4) the scorbutic diet with a daily supplement of 5 mg. of ascorbic acid per animal; (5) the scorbutic diet with methylthiouracil and a daily

---

[1] Desiccated thyroid tablets, Parke, Davis and Company, were used. 0.05 per cent refers to desiccated thyroid gland.

supplement of vitamin C; and (6) the scorbutic diet with thyroid and a daily supplement of vitamin C. The oral test for glucose tolerance was performed on the 22nd day in all the animals according to the method described previously (17). The results are given in Table I.

TABLE I

Glucose Tolerance Test in Guinea Pigs on Different Diets

The results are given in mg. per cent.

Diets fed*	Fasting blood sugar	Blood sugar after feeding glucose			
		45 min.	90 min.	135 min.	180 min.
Scorbutic diet only (8)	150 ± 2.2	326 ± 23.9	333 ± 22.4	306 ± 31.1	251 ± 37.1
" + methyl-thiouracil (8)	151 ± 10.7	298 ± 23.5	316 ± 31.2	333 ± 24.4	266 ± 37.2
Scorbutic + thyroid (8)	151 ± 5.3	331 ± 3.4	317 ± 17.0	251 ± 17.9	210 ± 16.4
" + vitamin C (8)	132 ± 5.4	279 ± 11.7	251 ± 17.2	194 ± 14.9	136 ± 4.1
Scorbutic + vitamin C and methylthiouracil (7)	121 ± 5.7	261 ± 10.8	256 ± 8.2	196 ± 12.6	146 ± 14.9
Scorbutic + vitamin C and thyroid (8)	147 ± 5.5	271 ± 10.5	262 ± 17.3	210 ± 20.2	170 ± 33.9

* The figures in the parentheses indicate the number of animals in the group.

SUMMARY

1. The types of glucose tolerance curves for each of the three groups of guinea pigs which received the scorbutic diet, the scorbutic diet with methylthiouracil, and the scorbutic diet with thyroid were more or less similar. This indicated that the deficient utilization of glucose observed in animals on the scorbutic diet only was not affected by either hypo- or hyperthyroidism.

2. The blood sugar level, 3 hours after feeding glucose to guinea pigs receiving thyroid gland and vitamin C, was significantly higher than that of the control animals receiving no thyroid gland. This indicated that hyperthyroidism lowers the carbohydrate tolerance in guinea pigs.

3. Methylthiouracil, however, had no effect on the glucose tolerance test, which showed that carbohydrate tolerance is not altered in hypothyroid guinea pigs.

4. The altered carbohydrate metabolism observed in scorbutic guinea pigs is, therefore, not due to either hypo- or hyperthyroidism.

BIBLIOGRAPHY

1. Geyelin, H. R., *Arch. Int. Med.*, **16**, 975 (1915).
2. Janney, N. W., and Isaacson, V. I., *Arch. Int. Med.*, **22**, 160 (1918).
3. Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, **18**, 87 (1914).
4. Feinblatt, H. M., *J. Lab. and Clin. Med.*, **8**, 500 (1923).
5. Houssay, B. A., *Semana méd.*, **1**, 255 (1944).
6. Andersen, W. T., *Acta med. Scand.*, **78**, 229 (1932).
7. Wilder, R. M., Foster, R. F., and Pemberton, J. de J., *Endocrinology*, **18**, 455 (1934).
8. Coggeshall, H. C., and Greene, J. A., *Am. J. Physiol.*, **105**, 103 (1933).
9. Prina, C., *Boll. Soc. ital. biol. sper.*, **22**, 949 (1946).
10. Sure, B., and Theis, R. M., *Proc. Soc. Exp. Biol. and Med.*, **37**, 646 (1938).
11. Demole, V., and Ippen, F., *Z. physiol. Chem.*, **235**, 226 (1935).
12. Svirbely, J. L., *J. Biol. Chem.*, **111**, 147 (1935).
13. Monetti, G., *Arch. studio fisiopatol. e clin. ricambio*, **9**, 63 (1941).
14. Lewis, R. A., *Bull. Johns Hopkins Hosp.*, **63**, 31 (1938).
15. Banerjee, S., and Ghosh, N. C., *J. Biol. Chem.*, **168**, 207 (1947).
16. Banerjee, S., *J. Biol. Chem.*, **159**, 327 (1945).
17. Banerjee, S., and Ghosh, N. C., *J. Biol. Chem.*, **166**, 25 (1946).

ENZYMATIC HYDROLYSIS OF SATURATED AND UNSATURATED TRIPEPTIDES

By PAUL J. FODOR,* VINCENT E. PRICE, and
JESSE P. GREENSTEIN

(*From the National Cancer Institute, National Institutes of Health,
Bethesda, Maryland*)

Previous studies on the relative rates of enzymatic hydrolysis by rat tissue extracts of analogous saturated and unsaturated dipeptides revealed the generally greater susceptibility of the L form of the former type of substrate (1). Thus, in the presence of liver preparations, glycyl-L-phenylalanine was hydrolyzed about 150 to 250 times faster than either glycyl-D-phenylalanine or glycyldehydrophenylalanine. In the case of certain pairs of analogous compounds, such as chloroacetyl-DL-phenylalanine and chloroacetyldehydrophenylalanine, the L form of the saturated peptide was rapidly attacked, whereas the D form and the dehydropeptide were apparently completely resistant.

It was considered of interest to extend these comparison studies to analogous saturated and unsaturated tripeptides. The saturated tripeptides have two peptide bonds, and the unsaturated tripeptides have one acyl peptide bond and one terminal dehydropeptide bond. In the case of tripeptides containing a racemic amino acid, four peptide bonds may be considered to coexist in the same substrates. We have therefore laid chief emphasis in this study on determining the maximum hydrolysis, under nearly identical conditions, of various related substrates after prolonged incubation periods in the presence of concentrated aqueous extracts of hog or rat kidney. In addition, a few rate studies have been made on selected substrates.

In the case of the dehydropeptides, the hydrolysis was followed either by measurement of the ammonia formed or by ultraviolet spectrophotometry (2), and therefore only the cleavage of the dehydropeptide bond was noted. There is little doubt, however, that, in the susceptible unsaturated tripeptides, both saturated and unsaturated peptide bonds are hydrolyzed. The enzymatic hydrolysis of the saturated dipeptides and tripeptides was followed by the manometric CO_2 procedure with ninhydrin; and therefore the cleavage of all susceptible peptide bonds in the substrates was noted.[1]

---

* Research Fellow, National Cancer Institute; on leave from The Hebrew University of Jerusalem.

[1] The dehydropeptides yield on hydrolysis not only ammonia but also the corre-

A number of analogous and isomeric peptides were prepared in order to interpret adequately the enzymatic data.

EXPERIMENTAL

Substrates[2]

The following compounds were prepared as described: glycyldehydroalanine (3), glycyl-L-alanine (4), glycyl-D-alanine (4), glycyl-DL-alanine (5), chloroacetyldehydroalanine (3), chloroacetyl-L-alanine (4), chloroacetyl-D-alanine (4), chloroacetyl-DL-alanine (5), acetyldehydroalanine (6, 7), acetyl-DL-alanine (4), glycyldehydrophenylalanine (8), glycyl-DL-phenylalanine (9), chloroacetyldehydrophenylalanine (8), chloroacetyl-DL-phenylalanine(9), acetyldehydrophenylalanine (10), acetyl-DL-phenylalanine (11), acetyldehydroleucine (12), acetyl-DL-leucine (4), sarcosyldehydroalanine (13), sarcosyldehydrophenylalanine (14), chloroacetylglycyldehydroalanine (14), glycylglycyldehydroalanine (14), chloroacetylglycyldehydrophenylalanine (15), glycylglycyldehydrophenylalanine (15), chloroacetyldehydrophenylalanylglycine (8),[3] glycyldehydrophenylalanylglycine (8),[3] chloroacetylsarcosyldehydroalanine (13), DL-alanylglycine (16), DL-phenylalanylglycine (17), glycylglycine (18), glycylglycylglycine (19), chloroacetylglycine (20), chloroacetylglycylglycine (20), chloroacetyl-DL-leucine (16), chloroacetylsarcosine (21), glycylsarcosine (21),[4] glycyl-DL-leucine (16), DL-leucylglycine (16), and DL-leucinamide (22). All the

---

sponding α-keto acids. Measurement of the hydrolysis of the saturated peptide bond in the tridehydropeptides by the ninhydrin procedure in the presence of appreciable amounts of α-keto acids presents considerable difficulty because of the small but variable amounts of carbon dioxide produced by decomposition of the keto acids (23).

[3] Many chloroacetylated amino acids and peptides do not crystallize from the reaction mixture after acidification with HCl. In such cases we have extracted the mixture several times with ethyl acetate, dried the combined extracts briefly over sodium sulfate, and evaporated the solvent at low temperature and pressure. The residue was washed several times with dry petroleum ether and finally treated with dry ether at −10°. The product invariably crystallized.

[3] The absorption spectrum in the ultraviolet of these compounds at 5 × 10⁻⁵ M concentration in water was practically identical with that of glycyldehydrophenylalanine or other aromatic dehydropeptides with an absorption maximum at 2750 A and molar extinction coefficient of 18,000 (13, 34).

[4] Our preparation possessed a melting point of 214° (uncorrected). Found C 40.8, H 7.2, N 18.8; calculated C 41.1, H 6.9, N 19.1 per cent. Levene et al. (21) reported a melting point of 200-201°. This lower melting point value might have been due to traces of ammonium chloride, for we have found that several recrystallizations from hot water-alcohol are necessary to free the peptide from this salt. The melting point of the preparation of glycylsarcosine made by the carbobenzoxy method by Bergmann et al. was 220° (corrected) (35).

compounds were recrystallized and characterized; the chloroacetylated compounds were free from chloride ions, and the aminated compounds free of ammonia and chloride.

Sarcosyl-DL-alanine—1 mole of chloroacetyl-DL-alanine was treated at 40° with 3 moles of methylamine in a 25 per cent aqueous solution. After 3 days, the solution was evaporated to a syrup *in vacuo*. The syrup was taken up in a small volume of water, treated with glacial acetic acid to pH 5.0, and a large volume of absolute alcohol was added. The product crystallized in long prisms. On recrystallization from water-alcohol the peptide melted at 182°. N found 17.3, calculated 17.5 per cent.

Sarcosyl-DL-phenylalanine—This compound·was prepared as above by the use of chloroacetyl-DL-phenylalanine and methylamine. Prisms. M.p. 191°; N found 11.5, calculated 11.8 per cent.

Chloroacetylglycyl-L-alanine—From glycyl-L-alanine (4) and chloroacetyl-chloride in NaOH solution. Recrystallized from absolute methanol as needles. M.p. 167°; N found 12.6, calculated 12.6 per cent.

$$[\alpha]_D^{25} = -42.7° \text{ (for 0.74\% solution in water)}$$

Chloroacetylglycyl-D-alanine—From glycyl-D-alanine (4) and chloroacetyl chloride in NaOH solution. Recrystallized from absolute methanol as needles. M.p. 167°; N found 12.3, calculated 12.6 per cent.

$$[\alpha]_D^{25} = +42.4° \text{ (for 1.09\% solution in water)}$$

Chloroacetylglycyl-DL-alanine—From glycyl-DL-alanine and chloroacetyl chloride in NaOH solution. Recrystallized from absolute methanol as needles. M.p. 163°; N found 12.4, calculated 12.6 per cent.

Glycylglycyl-DL-alanine—By amination of chloroacetylglycyl-DL-alanine in 10 times the volume of 28 per cent ammonia water for 24 hours at 40°. Recrystallized three times from water-alcohol. Long prisms. M.p. 228°; N found 20.5, calculated 20.7 per cent.

Chloroacetylglycyl-DL-phenylalanine—From glycyl-DL-phenylalanine and chloroacetyl chloride in NaOH solution. Recrystallized from ethyl alcohol as prisms. M.p. 151°; N found 9.2, calculated 9.4 per cent.

Glycylglycyl-DL-phenylalanine—By amination of chloroacetylglycyl-DL-phenylalanine in 10 times the volume of 28 per cent ammonia water for 24 hours at 40°. Recrystallized twice from water-alcohol as long prisms; m.p. 242°; N found 14.8, calculated 15.1 per cent.

Chloroacetyl-DL-phenylalanylglycine—From DL-phenylalanylglycine and chloroacetyl chloride in NaOH solution. Recrystallized from absolute methanol as long needles. M.p. 180°; N found 9.1, calculated 9.4 per cent.

Glycyl-DL-phenylalanylglycine—By amination of chloroacetyl-DL-phenylalanylglycine as above. Prisms from water-alcohol. M.p. 235°; N found 14.9, calculated 15.1 per cent.

*Chloroacetylsarcosyl-*DL-*alanine*—From sarcosyl-DL-alanine and chloroacetyl chloride in NaOH solution. Needles from absolute alcohol. M.p. 138°; N found 11.4, calculated 11.8 per cent. Attempts to aminate this compound, in the hope of obtaining glycylsarcosyl-DL-alanine, were unsuccessful.

Glycylsarcosyldehydroalanine—Attempts to aminate chloroacetylsarcosyldehydroalanine by use of aqueous ammonia were unsuccessful (13). A good preparation, however, was obtained by amination in ammoniacal absolute methanol. 10 gm. of chloroacetylsarcosyldehydroalanine (13) were dissolved in 100 cc. of absolute methanol which had been saturated at 0° with dry ammonia gas. After standing 5 hours at room temperature, the solution was evaporated *in vacuo* to a white solid mass which was taken up in hot water and treated with hot absolute alcohol in excess. The dehydropeptide was recrystallized twice more from water-alcohol as glistening prisms. Yield 38 per cent. M.p. 161°. Found C 41.2, H. 6.8, N 17.6 per cent; calculated for compound with 1 mole of crystal water C 41.2, H 6.9, N 18.0 per cent. When dried for 4 hours at 78° and 1 mm. pressure of mercury, the compound lost 7.5 per cent in weight; calculated for 1 mole of crystal water 7.7 per cent.[5]

*Chloroacetyl-*DL-*alanylglycine*—Prisms from absolute methanol. M.p. 146°; N found 12.4, calculated 12.6 per cent.

*Glycyl-*DL-*alanylglycine*—By amination of the above in aqueous ammonia. Prisms from water-alcohol. M.p. 242°; N found 20.6, calculated 20.7 per cent.

*Chloroacetyl-*DL-*leucylglycine*—Needles first from absolute methanol and then from water. M.p. 144°; N found 10.5, calculated 10.6 per cent.

*Chloroacetylglycyl-*DL-*leucine*—Needles first from absolute methanol and then from water. M.p. 160°; N found 10.6, calculated 10.6 per cent.

*Glycylglycyl-*DL-*leucine*—By amination of the above in aqueous ammonia. Prisms from water-alcohol. The compound did not melt when heated up to 240°. When heated at 78° and 1 mm. pressure of mercury for 4 hours, the compound lost 6.5 per cent in weight; calculated for 1 mole of crystal water 6.8 per cent. N found 17.1, calculated 17.1 per cent.

*Glycyl-*DL-*leucylglycine*—By amination of chloroacetyl-DL-leucylglycine in aqueous ammonia. Prisms from water-alcohol. The compound did not melt when heated to 240°. When heated at 78° and 1 mm. pressure of mercury for 4 hours, the compound lost 6.6 per cent in weight; calculated for 1 mole of crystal water 6.8 per cent. N found 17.0, calculated 17.1 per cent.

---

[5] The absorption spectrum in the ultraviolet of this compound at 1.7×10^{-4} M concentration in water was practically identical with that of glycyldehydroalanine or other aliphatic dehydropeptides with an absorption maximum at 2400 A and molar extinction coefficient of 4450 (3, 13).

Chloroacetyl-N-methyl-DL-alanine—1 mole of α-chloropropionic acid was treated with 10 moles of 33 per cent methylamine solution for 2 days at 40°. The solution was evaporated to a syrup *in vacuo*, taken up in a small amount of water, treated with glacial acetic acid to pH 5.0, and finally treated with the 10-fold volume of absolute alcohol. After standing for 24 hours at −10°, the N-methyl-DL-alanine was filtered, washed with alcohol, and recrystallized from water-alcohol. Yield 44 per cent. N found 13.5, calculated 13.6 per cent. On chloroacetylation in the usual manner with chloroacetyl chloride in NaOH solution, chloroacetyl-N-methyl-DL-alanine was obtained in 64 per cent yield. Long needles from hot absolute alcohol. M.p. 82°; N found 7.8, calculated 7.8 per cent.

Glycyl-N-methyl-DL-alanine—By amination of the above compound in a 10-fold volume of 28 per cent aqueous ammonia for 3 days at 40°. After evaporation of the ammonia solution *in vacuo*, the residue was taken up in the minimum amount of cold water and treated with an excess of alcohol. On standing 1 week at −10°, long prisms separated. Yield 28 per cent. The product was recrystallized from hot water-alcohol. M.p. 155°; found C 45.0, H 7.8, N 17.3, calculated C 45.0, H 7.5, N 17.5 per cent.

Chloroacetyl-DL-leucinamide—2.6 gm. of DL-leucinamide were dissolved in 15 cc. of water and chilled. The amide was then chloroacetylated in the usual manner with 2.6 gm. of chloroacetyl chloride, and 10 cc. of 2 N NaOH were added alternately with shaking and with chilling. The product which crystallized during the reaction was filtered at the pump and washed with cold water. It was recrystallized from the minimum amount of absolute methanol from which it separated as flat plates. M.p. 155°; found C 46.5, H 7.6, N 13.3, calculated C 46.5, H 7.3, N 13.5 per cent.

Enzymatic Procedure

The digests were composed of 1 cc. of either hog or rat kidney aqueous extract (prepared by grinding the tissue with sand, taking up the paste in distilled water, and lightly centrifuging), 1 cc. of 0.15 M borate buffer at pH 8.0 or 1 cc. of 0.07 M phosphate buffer at pH 7.2, and 1 cc. of either water or neutralized substrate solution. A concentration of 0.05 M was employed for the racemic substrates, and 0.025 M for all the others. The concentration of extract and the period of incubation at 37° were varied to suit the criterion of nearly maximum hydrolysis. No activators were employed at this time with the crude extract preparations. In all cases the determinations were continued either until (a) the hydrolysis had practically reached an end-point, (b) the titer was so negligible as to raise doubt as to whether any hydrolysis at all had occurred, or (c) until the hydrolysis of relatively resistant bonds in certain substrates was measurable but so

slow as to render impracticable further incubation of the digest. The longest period of incubation for any substrate was 8 hours.[6]

The hydrolysis of the dehydropeptides, with the exception of that of glycylsarcosyldehydroalanine, was measured in terms of the ammonia produced in the digests (2). The hydrolysis of glycylsarcosyldehydroalanine
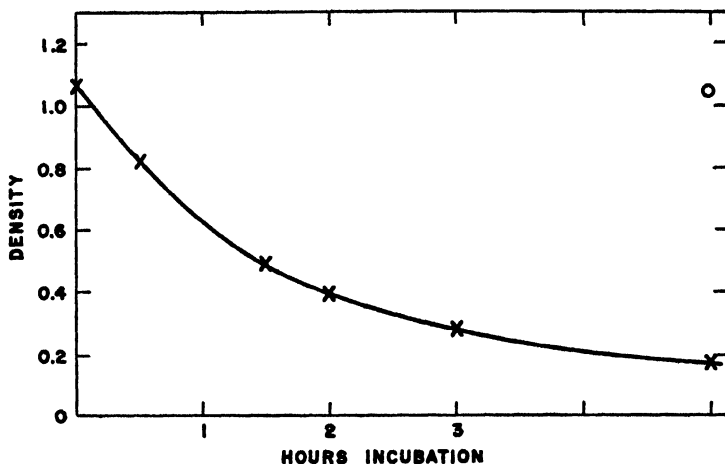
FIG. 1. Hydrolysis of glycylsarcosyldehydroalanine at 37°. The digests consisted of 1 cc. of hog kidney aqueous extract equivalent to 660 mg. of fresh tissue, 2 cc. of 0.15 M borate buffer at pH 8.1, and 1 cc. of 0.025 M substrate. The reaction was followed spectrophotometrically at 2400 A; the cells were used with a 1 cm. path. O, substrate solution in the presence of the buffer.

could not be readily measured by this procedure, for, unlike all the other dehydropeptides studied, this compound was unstable in the presence of the saturated potassium carbonate solution employed for the aeration of the evolved ammonia. The alternative spectrophotometric procedure was

[6] It is probable in several instances that, had the incubation period been extended well beyond the period chosen, the relatively resistant bonds would have been completely hydrolyzed, and perhaps even those apparently completely resistant bonds in certain substrates might have been at least measurably attacked. It is sometimes judicious, however, to set some sort of a limit to what an enzyme might reasonably be expected to do, and we are reluctant to ascribe authentic enzymatic activity to a reaction which, under otherwise optimum conditions, requires an undue amount of time. We have generally set, as the lower limit of enzymatic activity in hydrolytic reactions involving the saturated or unsaturated peptide bond, a rate value of 0.1 μM of substrate hydrolyzed per hour per mg. of protein N. Any apparent rate of cleavage less than this we consider dubious. Since relatively dilute extract concentrations and short incubation periods were used with highly susceptible racemic substrates from which D-amino acids were hydrolyzed, no concern over oxidase action on these latter acids was taken. The quantitative carboxyl nitrogen titer in such digests confirmed the lack of appreciable oxidase action.

therefore employed to follow the hydrolysis of this substrate (progressive decrease in absorption at 2400 A) (2); (Fig. 1). The hydrolysis of the saturated peptides was measured in terms of the CO_2 evolved during the ninhydrin procedure of Van Slyke *et al.* (23).

All the determinations were corrected for extract and substrate blanks. Under the conditions used, the latter were usually quite negligible. The liberation of each amino acid from the optically active peptides and of ammonia from the dehydropeptides gives rise to 25 μM of COOH-N and
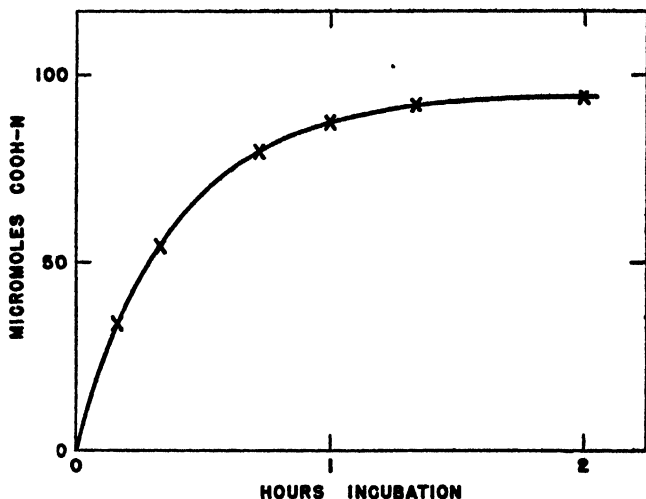
FIG. 2. Hydrolysis of glycyl-DL-phenylalanylglycine at 37°. The digests consisted of 1 cc. of rat kidney aqueous extract equivalent to 33 mg. of fresh tissue, 1 cc. of 0.15 M borate buffer at pH 8.1, and 1 cc. of either water or 0.05 M substrate solution.

of NH_3-N respectively. Thus, the complete hydrolysis of glycylglycyl-DL-alanine and of glycylglycyldehydroalanine yields 150 μM of COOH-N and 25 μM of NH_3-N respectively. The procedure is illustrated by the hydrolysis of glycyl-DL-phenylalanylglycine given in Fig. 2. A maximum of 100 μM of COOH-N is liberated from 50 μM of this substrate. It may be presumed that the glycyl-L-phenylalanylglycine component of the racemate is completely hydrolyzed, accounting for 75 μM of COOH-N, while the other 25 μM are derived by hydrolysis of the acyl glycine from glycyl-D-phenylalanylglycine. The residual D-phenylalanylglycine is relatively resistant to enzymatic hydrolysis (see below).

Results

The effect of pH on the enzymatic hydrolysis of several types of saturated and unsaturated peptides is described in Figs. 3 and 4. The pH
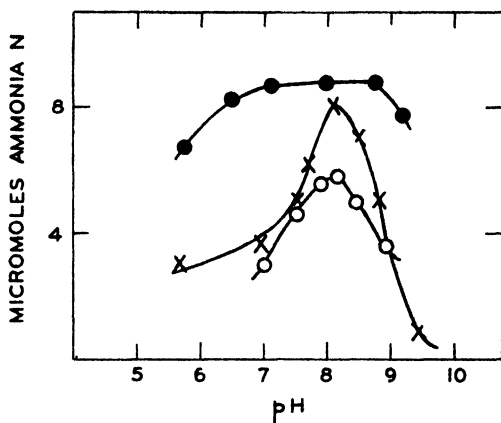
FIG. 3. Effect of pH on hydrolysis of dehydropeptides at 37°. The digests consisted of 1 cc. of hog kidney aqueous extract, 2 cc. of buffer, and 1 cc. of either water or 0.025 M neutralized substrate. ×, chloroacetylglycyldehydroalanine, ○, glycyldehydroalanine, ●, chloroacetyldehydroalanine. Extract concentrations were adjusted for each substrate so as to yield a linear rate of hydrolysis. The veronal buffer was used below pH 8.0, the glycine-NaOH buffer above pH 8.0.
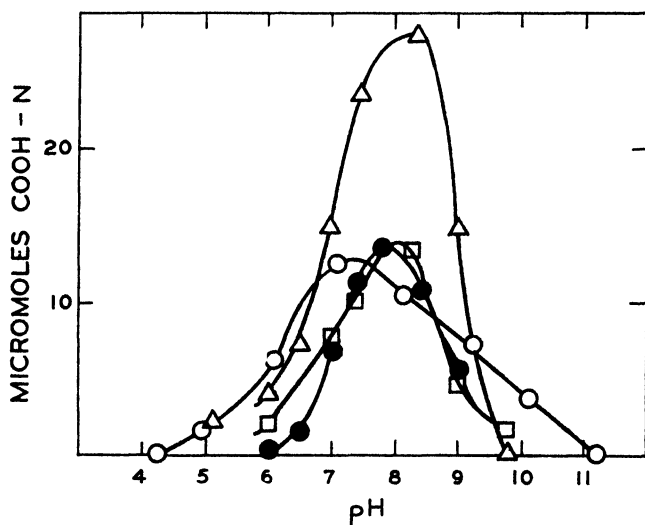
FIG. 4. Effect of pH on hydrolysis of saturated peptides at 37°. Experimental conditions were similar to those described in Fig. 3, except that 0.05 M neutralized substrate solutions and only 1 cc. of buffer were used. □, chloroacetylglycyl-DL-alanine, ●, glycyl-DL-alanine, ○, chloroacetyl-DL-alanine, △, chloroacetyl-DL-alanylglycine. The curve for chloroacetylglycine resembles that for chloroacetyl-DL-alanine.

activity curve for chloroacetyldehydroalanine is similar to that obtained in rat liver digests (14), and is quite different in shape from that obtained with chloroacetyl-DL-alanine. The dehydropeptide is hydrolyzed to nearly the same extent from pH 6.5 to 9.0. The analogous saturated peptide is

TABLE I

Action of Concentrated Hog Kidney Aqueous Extract on Analogous Saturated and Unsaturated Peptides at Nearly Maximum Hydrolysis

Substrate	Extract concentration	Incubation period	NH₂-N or -COOH-N liberated	Peptide bonds	
				Available	Hydrolysed
	mg. N per cc.	*hrs.*	*μM*		
Glycyldehydroalanine	2.0	1	24		
Glycyl-L-alanine	2.3	2	50	1	1
Glycyl-D-alanine	2.3	2	50	1	1
Chloroacetyldehydroalanine	2.1	4	25		
Chloroacetyl-L-alanine	0.1	1	25	1	1
Chloroacetyl-D-alanine	2.1	8	0	1	0
Acetyldehydroalanine	2.1	4	24		
Acetyl-DL-alanine	0.2	2	25	2	1
Glycyldehydrophenylalanine	2.1	1	25		
Glycyl-DL-phenylalanine	2.3	4	100	2	2
Chloroacetyldehydrophenylalanine	2.3	8	0		
Chloroacetyl-DL-phenylalanine	0.7	2	25	2	1
Acetyldehydrophenylalanine	2.1	8	0		
Acetyl-DL-phenylalanine	3.8	4	25	2	1
Acetyldehydroleucine	3.9	6	0		
Acetyl-DL-leucine	0.3	1	25	2	1
Sarcosyldehydroalanine*	2.3	2	24		
Sarcosyl-DL-alanine*	2.3	4	100	2	2
Sarcosyldehydrophenylalanine*	2.3	4	25		
Sarcosyl-DL-phenylalanine*	2.4	4	100	2	2
Chloroacetylglycyldehydroalanine	2.2	8	24		
Chloroacetylglycyl-L-alanine	2.2	4	50	2	2
Chloroacetylglycyl-D-alanine	2.2	4	0	2	0
Glycylglycyldehydroalanine	1.0	1	25		
Glycylglycyl-DL-alanine	2.1	2	150	4	4
Chloroacetylglycyldehydrophenylalanine	2.4	8	0		
Chloroacetylglycyl-DL-phenylalanine	2.4	4	50	4	2
Glycylglycyldehydrophenylalanine	2.4	1	25		
Glycylglycyl-DL-phenylalanine	2.6	4	150	4	4
Chloroacetyldehydrophenylalanylglycine	3.8	6	0		
Chloroacetyl-DL-phenylalanylglycine	2.9	2	50	4	2
Glycyldehydrophenylalanylglycine	2.2	8	0†		
Glycyl-DL-phenylalanylglycine	2.4	4	85	4	2+‡§

TABLE I—*Concluded*

Substrate	Extract concentration	Incubation period	NH₂-N or —COOH-N liberated	Peptide bonds	
				Available	Hydrolyzed
	mg. N per cc.	*hrs.*	*µM*		
Chloroacetylsarcosyldehydroalanine	2.4	6	0		
Chloroacetylsarcosyl-DL-alanine... ...	2.9	4	0	4	0
Glycylsarcosyldehydroalanine	2.4	8	24		
DL-Alanylglycine..	2.4	2	73.1	2	1+‡
DL-Phenylalanylglycine	2.4	4	60.3	2	1+‡
Chloroacetyl-DL-alanylglycine. . .	2.9	4	50	4	2
Glycyl-DL-alanylglycine.	2.2	4	112	4	3+‡
Glycylglycine	3.0	2	50	1	1
Glycylglycylglycine....	3.0	2	75	2	2
Chloroacetylglycine....	0.5	1	25	1	1
Chloroacetylglycylglycine..... . .	2.6	4	31	2	1+‡
Chloroacetylsarcosine..... . . .	3.0	4	0	1	0
Glycylsarcosine	3.0	4	44	1	0+‖
Glycyl-DL-leucine	2.3	2	100	2	2
Chloroacetyl-DL-leucine. .. .	2.3	2	25	2	1
DL-Leucylglycine	2.4	4	67.1	2	1+‡
Chloroacetylglycyl-DL-leucine. . ..	2.4	4	35.5	4	1+‡
Chloroacetyl-DL-leucylglycine	3.8	4	50	4	2
Glycylglycyl-DL-leucine... .	2.4	2	150	4	4
Glycyl-DL-leucylglycine. . . .	3.8	4	83	4	2+‡
Chloroacetyl-N-methyl-DL-alanine . .	2.3	4	0	2	0
Glycyl-N-methyl-DL-alanine .. .	3.8	8	25	2	0+‖

A plus sign indicates that the bond is seemingly very slowly hydrolyzed.

* These sarcosyl peptides and dehydropeptides were hydrolyzed at a markedly slower rate than the corresponding glycyl forms.

† Not hydrolyzed either by rat kidney extracts.

‡ Incomplete hydrolysis of another peptide bond.

§ With rat kidney, close to 100 µM of COOH-N is liberated (Fig. 2), or equivalent to the hydrolysis of three peptide bonds.

‖ Incomplete hydrolysis of one peptide bond.

hydrolyzed with an optimum at about pH 7.2. Only chloroacetyldehydro-alanine and chloroacetyl-DL-alanine differ in this respect, for glycyldehydro-alanine, glycyl-DL-alanine, chloroacetylglycyldehydroalanine, chloroacetyl-glycyl-DL-alanine, and chloroacetyl-DL-alanylglycine are all hydrolyzed with an optimum rate at about pH 8.0 to 8.2.

The effect of incubating a relatively large series of saturated and un-saturated peptides under nearly optimum conditions, until all of the readily hydrolyzable bonds are cleaved, is described in Table I.

When it is reasonably clear that saturated peptide bonds are or are not hydrolyzed, the data are given in the form of whole numbers. When one bond is seemingly very slowly hydrolyzed, a plus sign follows the number of hydrolyzable bonds described in order to indicate the presence of this relatively resistant bond. In the case of the dehydropeptides, the hydrolysis of the single dehydropeptide bond occurred either practically completely or not at all.

Rates of Hydrolysis of Certain Peptides and Dehydropeptides—In Table II are given the values of initial reaction rates of some of the susceptible substrates with hog kidney extracts. In all cases, the rates of hydrolysis for

TABLE II

Rates of Hydrolysis of Analogous Peptides and Dehydropeptides with Aqueous Hog Kidney Extracts*

Substrate	Substrate hydrolyzed
	μM per hr. per mg. N
Chloroacetyldehydroalanine. .	33
Chloroacetyl-L-alanine	1161
Glycyldehydroalanine	237
Glycyl-L-alanine . . .	2860
Glycyl-D-alanine .	120
Glycyl-N-methyl-DL-alanine	2
Chloroacetylglycine	133
Glycylglycine	1765
Glycylsarcosine	2
Chloroacetylglycyldehydroalanine	1
Chloroacetylglycyl-L-alanine	25
Glycylglycyldehydroalanine	210
Glycylsarcosyldehydroalanine . .	2

* All the reactions were conducted at pH 8.0, except in the cases of chloroacetyl-L-alanine and chloroacetylglycine, which were carried out at pH 7.2.

the respective compounds are considerably higher with hog kidney than with rat kidney extracts (14). Chloroacetyldehydroalanine is hydrolyzed at a rate about 30 times slower than chloroacetyl-L-alanine, while glycyldehydroalanine is hydrolyzed about 10 times more slowly than glycyl-L-alanine and about twice as fast as glycyl-D-alanine. Glycyl-N-methyl-DL-alanine and glycylsarcosine are hydrolyzed extremely slowly; the latter compound is completely resistant to the action of intestinal mucosa under conditions whereby glycyl-L-proline is readily hydrolyzed (24). In accord with the relative resistance of the glycylsarcosine bond compared with the glycylglycine bond, the susceptibility of glycylglycyldehydroalanine is greater than that of glycylsarcosyldehydroalanine. Chloroacetylglycylde-

hydroalanine is hydrolyzed at a rate about 25 times slower than chloro-acetylglycyl-L-alanine, a ratio which is of the same order of magnitude as that for the rates of hydrolysis for chloroacetyldehydroalanine and chloro-acetylglycyl-L-alanine.

Rough estimations of the rate of hydrolysis of the isomeric chloro-acetylated dipeptides revealed that those substrates were more susceptible which possessed glycine as the terminal residue. Thus, under identical experimental conditions, with a hog kidney aqueous extract equivalent to 166 mg. of fresh tissue per cc., the following values in micrograms of CO_2 were obtained from digests of 50 μM of substrate after 2 hours of incubation at 37°: chloroacetylglycyl-DL-phenylalanine 234, chloroacetyl-DL-phenyl-alanylglycine 600 (maximum value); and chloroacetylglycyl-DL-alanine 224, chloroacetyl-DL-alanylglycine 505. With a more concentrated extract prep-aration and an 8 hour period of incubation both chloroacetylglycyl-DL-phenylalanine and chloroacetylglycyl-DL-alanine were hydrolyzed at all peptide bonds.

<div align="center">DISCUSSION</div>

Study of the enzymatic susceptibility of acylated amino acids and pep-tides possesses a special interest in the present series of investigations. The intracellular N-acylase systems, first described under the designation "histozyme" (25) and applied almost exclusively to N-benzoylated amino acids such as hippuric acid, have received relatively little attention. In-terest in this laboratory was early directed to this topic when it was noted that the dehydropeptides, acetyldehydroalanine and chloroacetyldehydro-alanine, were hydrolyzed by kidney and liver extracts, and that the activity toward these substrates could be readily separated from the activity toward glycyldehydroalanine (2).

Similar studies on analogous saturated peptides revealed the presence of enzyme systems in rat and hog kidney which acted asymmetrically upon such substrates as chloroacetyl-DL-alanine (1, 26) and permitted thereby the development of a method of preparing L- and D-amino acids in quantity and in a state of high optical purity (4).

There appear to be systems in hog kidney capable of acting upon tripep-tides lacking an α-amino group on the acyl residue, such as chloroacetyl-glycyldehydroalanine, chloroacetylglycyl-L-alanine, or chloroacetyl-L-al-anylglycine (Table I). These enzymes possess at least two apparently absolute specificities: (a) The acyl-saturated peptide bond, whether in the completely saturated tripeptide or in the tripeptide with one acyl-saturated and one terminal-unsaturated peptide bond, must be ordinarily susceptible, e.g. chloroacetylsarcosyl-L-alanine, chloroacetylsarcosyldehydroalanine, chloroacetyl-D-phenylalanylglycine, or chloroacetyldehydrophenylalanyl-

glycine is not attacked; and (b) the acyl and terminal residues in the tripeptide chain must possess such a structure or configuration that, if they were united in dipeptide combination, they would be enzymatically susceptible; e.g., whereas chloroacetylglycyl-L-alanine and chloroacetylglycyl-dehydroalanine are hydrolyzed, chloroacetylglycyl-D-alanine and chloroacetylglycyldehydrophenylalanine are completely resistant (Table I). Thus, the susceptibility of the acylated dipeptides is governed by the relation between the acyl and intermediate residues and between the acyl and terminal residues, and is not apparently concerned with the relation between the intermediate and terminal residues.[7]

The susceptibility of certain of the acylated amino acids and peptides by hog kidney invites comparison with the carboxypeptidase system of the pancreas. Both carboxypeptidase and the hog kidney systems act upon peptides lacking a free α-amino group on the acyl residue. The former acts much more readily on those substrates whose terminal amino acid residues are tyrosine, tryptophan, or phenylalanine, and much less readily when such residues are glycine, alanine, or other aliphatic amino acids (27–30). The reverse is true for the hog kidney systems (2, 4). The initial attack of pancreatic carboxypeptidase on tripeptides is on the terminal bond; that for the hog kidney systems is not definitely known. The fact that hog kidney hydrolyzes chloroacetylglycyl-L-alanine but not chloroacetylsar-cosylalanine' would suggest that the primary point of attack is at the acyl peptide bond. On the other hand, the fact that the hog kidney hydrolyzes chloroacetylglycyl-L-alanine but not chloroacetylglycyl-D-ala-nine would suggest with equal cogency that the initial point of attack was at the terminal peptide bond. Unquestionably, the configuration about each of the bonds in these substrates is important, and the question of the initial point of attack of the hog kidney systems on the acylated dipeptides may well be left open at this time.

Both saturated and unsaturated peptides with a free α-amino group on the acyl residue are much more susceptible to the action of kidney systems than those corresponding substrates lacking the α-amino group. In the

[7] Fractionation studies on hog kidney extracts at present being actively pursued in this laboratory have suggested that the enzymes acting upon certain dipeptides and tripeptides are different entities. A hog kidney aqueous extract from which the nuclear fraction was removed by light centrifugation was subjected to 26,000 × g in a refrigerated International centrifuge for 1 hour. The activity toward chloro-acetylglycine, chloroacetyl-L-alanine, chloroacetyldehydroalanine, and glycyl-L-alanine was found very largely in the supernatant. The activity toward chloro-acetylglycyl-L-alanine, chloroacetylglycyldehydroalanine, and glycyldehydroalanine was found very largely in the sediment. Similarly, fractionation studies with alcohol precipitations at very low temperatures have yielded results in the same direction. These studies will form the basis of a future communication.

case of the tripeptides, there is little doubt that the primary point of attack is the aminoacyl peptide bond, which is the characteristic of the classic aminopeptidase systems. Thus, the order of magnitude of the hydrolysis rate of glycylsarcosyldehydroalanine is close to that of glycylsarcosine (Table II).

There are apparently no enzymes in hog kidney capable of acting upon acylated D-amino acids, but there are enzymes capable of acting upon amino-acylated D-amino acids as glycyl-D-alanine, etc. On the other hand, those isomeric dipeptides in which the acylamino acid is of the D configuration and the terminal residue is glycine, such as D-leucylglycine, etc., are hydro-lyzed extremely slowly if at all (31). This latter fact accounts for the failure to hydrolyze readily all of the bonds in such tripeptides as glycyl-D-leucylglycine, although the isomeric tripeptide, glycylglycyl-D-leucine, is hydrolyzed completely.

Three of the four peptide bonds of glycyl-DL-phenylalanylglycine are readily hydrolyzed by rat kidney extracts (Fig. 2). On the other hand, the analogous tripeptide, glycyldehydrophenylalanylglycine, is completely resistant (15).[8] It would appear that none of the peptidases capable of acting upon the saturated peptide acts upon the analogous dehydropeptide. Chloroacetyl-L-phenylalanylglycine and acetyl-L-leucine are completely sus-ceptible substrates, whereas chloroacetyldehydrophenylalanylglycine (15) (Table I) and acetyldehydroleucine (32) are completely resistant. Taken together with recent evidence on the separation by high speed centrifuga-tion of the activities toward glycyl-L-alanine, glycyl-D-alanine, and glycyl-dehydroalanine, respectively (33), it would appear that peptidases and de-hydropeptidases were distinct and sometimes separable entities.

The authors are indebted to Mr. Robert Koegel for the nitrogen analyses.

SUMMARY

1. A considerable number of dipeptides and tripeptides, some of them containing all-saturated and some containing unsaturated peptide bonds, were incubated with hog or rat kidney aqueous extracts under conditions whereby nearly the maximum hydrolysis of the susceptible bonds were achieved.

2. Like chloroacetyldehydroalanine, chloroacetyl-L-alanine, chloroacetyl-

---

[8] The lower rate of hydrolysis of the acyl bond between glycine and D-phenyl-alanylglycine in glycyl-D-phenylalanylglycine by hog kidney is the only example so far noted of a weaker activity in this tissue than in rat kidney. The difference may be due (a) to a natural property of the kidneys of the two species, or (b) to the fact that the hog kidney was frozen before use, whereas the rat kidney was employed fresh.

L-phenylalanine and chloroacetyl-L-leucine, the tripeptides, chloroacetyl-glycyldehydroalanine, chloroacetylglycyl-L-alanine, chloroacetylglycyl-L-phenylalanine, and chloroacetylglycyl-L-leucine, are completely hydrolyzed. Again, like chloroacetyldehydrophenylalanine, chloroacetyl-D-alanine, chloroacetyl-D-phenylalanine, and chloroacetyl-D-leucine, the tripeptides, chloroacetylglycyldehydrophenylalanine, chloroacetylglycyl-D-alanine, etc., are practically completely resistant to enzymatic action of hog kidney. In as much as the dipeptides themselves, such as glycyldehydrophenyl-alanine and glycyl-D-alanine, are readily hydrolyzed, it would appear that the susceptibility of the chloroacetylated dipeptides and dehydropeptides was governed largely by the nature of the acyl and terminal residues.

3. The pH-activity curve for chloroacetyldehydroalanine shows a broad optimum zone between pH 6.5 and 9. That for chloroacetyl-L-alanine has a relatively sharp optimum at pH 7.2. The pH-activity curves for chloro-acetylglycyldehydroalanine, glycyldehydroalanine, chloroacetylglycyl-L-al-anine, glycyl-L-alanine, and chloroacetyl-DL-alanylglycine all show an opti-mum at about pH 8.0.

4. Chloroacetyl-DL-alanylglycine, chloroacetyl-DL-leucylglycine, and chloroacetyl-DL-phenylalanylglycine are half hydrolyzed, and it is pre-sumed that it is the L moieties of these racemic tripeptides which are sus-ceptible. Chloroacetyldehydrophenylalanylglycine is completely resistant.

5. The glycylglycylated DL-amino acids and glycylglycylated dehydro-amino acids are completely hydrolyzed, probably through the primary action of aminopeptidase on the susceptible aminoacyl bond. Glycyl-sarcosyldehydroalanine is slowly hydrolyzed, in accord with the low degree of susceptibility of the glycylsarcosine bond. The isomeric tripeptides, such as glycyl-DL-alanylglycine, glycyl-DL-phenylalanylglycine, and glycyl-DL-leucylglycine, are hydrolyzed at three or close to three bonds, instead of the theoretical four. The reason for this presumably lies in the relative resistance of the bond between the D-amino acid and the terminal glycine residue. Such dipeptides as D-alanylglycine are hydrolyzed extremely slowly. Glycyldehydrophenylalanylglycine is completely resistant, a phe-nomenon which at once sets off the dehydropeptidase from the peptidases which act upon the analogous saturated peptides.

6. Approximate rate studies were made with several types of substrates. The aminoacylated substrates are hydrolyzed at a faster rate than the corresponding chloroacetylated substrates, whether dipeptides or tripep-tides, or containing saturated or unsaturated bonds. The peptides are in general hydrolyzed at a faster rate than the dehydropeptides, except for glycyl-D-alanine which is cleaved at a slower rate than glycyldehydroalanine. Hydrolysis of chloroacetyl-DL-phenylalanylglycine and of chloroacetyl-DL-alanylglycine was considerably faster than that, respectively, of the

isomeric substrates, chloroacetylglycyl-DL-phenylalanine and chloroacetyl-glycyl-DL-alanine. It is suggested that different enzyme entities act upon dipeptides and tripeptides.

BIBLIOGRAPHY

1. Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **175**, 969 (1948).
2. Greenstein, J. P., *Advances in Enzymol.*, **8**, 117 (1948).
3. Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **171**, 477 (1947).
4. Fodor, P. J., Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **178**, 503 (1949).
5. Fischer, E., *Ber. chem. Ges.*, **37**, 2486 (1904).
6. Bergmann, M., and Grafe, K., *Z. physiol. Chem.*, **187**, 187 (1930).
7. Gonçalves, J. M., Price, V. E., and Greenstein, J. P., *J. Nat. Cancer Inst.*, **7**, 443 (1947).
8. Bergmann, M., Schmitt, V., and Miekeley, A., *Z. physiol. Chem.*, **187**, 264 (1930).
9. Leuchs, H., and Suzuki, U., *Ber chem Ges.*, **37**, 3306 (1904).
10. *Org. Syntheses*, coll. **1** (1944).
11. Knoop, F., and Blanco, J. G., *Z. physiol. Chem.*, **146**, 267 (1925).
12. Doherty, D. G., Tietzman, J. E., and Bergmann, M., *J. Biol. Chem.*, **147**, 617 (1943).
13. Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **173**, 337 (1948).
14. Greenstein, J. P., Price, V. E., and Leuthardt, F. M., *J. Biol. Chem.*, **175**, 953 (1948).
15. Bergmann, M., and Schleich, H., *Z. physiol. Chem.*, **205**, 65 (1932).
16. Fischer, E., *Ann. Chem.*, **340**, 123 (1905).
17. Fischer, E., *Ber. chem. Ges.*, **37**, 3062 (1904).
18. Fischer, E., and Fourneau, E., *Ber. chem. Ges.*, **34**, 2868 (1901).
19. Fischer, E., *Ber. chem. Ges.*, **36**, 2982 (1903).
20. Fischer, E., and Otto, E., *Ber. chem. Ges.*, **36**, 2106 (1903).
21. Levene, P. A., Simms, H. S., and Pfaltz, M. H., *J. Biol. Chem.*, **61**, 445 (1924).
22. Bergell, P., and Wülfing, H. V., *Z. physiol. Chem.*, **64**, 348 (1909).
23. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).
24. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **117**, 189 (1937).
25. Neuberg, C., *Biochem. Z.*, **147**, 372 (1924); also for review of earlier literature.
26. Waldschmidt-Leitz, E., Enzyme actions and properties, New York, 2nd edition, 168-169 (1929).
27. Waldschmidt-Leitz, E., and Klein, W., *Ber. chem. Ges.*, **61**, 640 (1928).
28. Stahmann, M. A., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **164**, 753 (1946).
29. Fruton, J. S., in Green, D. E., Currents in biochemical research, New York, 123-125 (1946).
30. Elkins-Kaufman, E., and Neurath, H., *J. Biol. Chem.*, **175**, 893 (1948).
31. Maschmann, E., *Biochem. Z.*, **313**, 129 (1943).
32. Price, V. E., and Greenstein, J. P., *Arch. Biochem.*, **18**, 383 (1948).
33. Price, V. E., Meister, A., and Greenstein, J. P., *Federation Proc.*, **8**, 238 (1949).
34. Yudkin, W. H., and Fruton, J. S., *J. Biol. Chem.*, **169**, 521 (1947).
35. Bergmann, M., Zervas, L., Schleich, H., and Leinert, F., *Z. physiol. Chem.*, **212**, 72 (1932).

EFFECT OF ANIONS ON THE NON-ENZYMATIC DESAMIDATION OF GLUTAMINE

By JAMES B. GILBERT, VINCENT E. PRICE, AND
JESSE P. GREENSTEIN

(*From the National Cancer Institute, National Institutes of Health,
Bethesda, Maryland*)

Glutaminase is the designation given the enzyme which catalyzes the hydrolysis of glutamine at the amide bond to yield as products ammonia and glutamic acid (1). Highly specific preparations of this enzyme from dog and beef kidney have been prepared (2). The enzymatic desamidation of glutamine in aqueous extracts of rat and mouse tissues is considerably accelerated by added phosphate, arsenate, and sulfate (3–6). The enzyme which is activated by these anions, and which is relatively heat-labile, acid-labile, and sedimentable on high speed centrifugation of the extract, has been given the designation of glutaminase I (5, 6).[1] Under the conditions of the enzymatic reaction (1 hour incubation at 37° at pH 8.0 in the presence of 14 μM of glutamine, 330 mg. of fresh tissue, and 0.01 to 0.20 M phosphate), whereby the substrate is nearly completely desamidated, less than 5 per cent of the substrate is hydrolyzed when the fresh tissue is replaced by boiled tissue.[2]

The amide group of glutamine is unusually labile at higher temperatures, and numerous methods for the determination of this compound in biological materials have been based upon this property (7–11). Thus, solutions of glutamine heated at 100° for several hours readily yield ammonia and pyrrolidonecarboxylic acid (7–10). Hamilton has shown that this conversion occurs more rapidly in the presence of phosphate than of citrate, acetate, or lactate solutions at the same molar concentration (11).

In view of this observation of Hamilton's, the question naturally arises as to whether the enzyme simply accelerates still further a desamidation reaction already catalyzed by phosphate. It was therefore considered desirable to investigate the effect of several anions on the non-enzymatic desamidation of glutamine, including both those anions which accelerate and those which do not accelerate the enzymatic desamidation of glutamine.

---

[1] The designation of glutaminase II has been given to the soluble, hepatic system which hydrolyzes glutamine only in the presence of α-keto acids (5, 14).

[2] Phosphate-activated glutaminase activity is demonstrated not only in tissue extracts but in slices as well (15).

The glutamine employed gave no test for ammonia. On boiling in 1 N HCl for 1 minute it yielded 90 per cent of the theoretical amount of amide nitrogen as ammonia. The non-enzymatic digests consisted of 1 cc. of glutamine at the stated concentration, 2 cc. of veronal acetate buffer (0.028 M with respect to veronal), and 1 cc. of either water or the anion at the stated concentration. The pH of each digest was carefully adjusted, and initial and final pH values were nearly identical. The enzymatic digests consisted of 1 cc. of fresh aqueous rat liver extract equivalent to 330 mg. of tissue, 2 cc. of the veronal buffer with and without added anion, and 1 cc. of either water or 0.014 M glutamine solution. At the end of the reaction, the digests were alkalinized with saturated K_2CO_3 and the ammonia aerated into dilute sulfuric acid traps and subsequently nesslerized. The glutamine was entirely stable under the aeration conditions. The enzymatic data were corrected for the extract blanks. Determinations of carboxyl nitrogen were made by the gasometric procedure with ninhydrin (12).

The anions were furnished by the following sodium salts: phosphate, phosphite, arsenate, methyl arsenate, bicarbonate, sulfate, borate, pyruvate, acetate, chloride, and nitrate.

Time Course of Non-Enzymatic Desamidation of Glutamine—The very considerable effect of phosphate on the non-enzymatic desamidation of glutamine is revealed in Fig. 1. After 48 hours of incubation, when nearly 100 per cent of the glutamine in the presence of phosphate was desamidated, only about 30 per cent of the amide in the absence of this anion was desamidated.

Effect of Various Anions on Non-Enzymatic Desamidation of Glutamine—The effect of increasing amounts of various sodium salts on the liberation of amide nitrogen from glutamine is revealed in Fig. 2. The values given are corrected for the ammonia evolved from the digests in the absence of added salt. At equimolar concentrations, phosphate was more effective than arsenate, and arsenate more so than methyl arsenate. Phosphite had a relatively weak effect. Borate accelerated the non-enzymatic desamidation of glutamine, but this salt was too insoluble to be used much above 0.05 M final concentration. Chloride, sulfate, nitrate, acetate, and pyruvate up to 0.20 M final concentration possessed no measurable effect on the desamidation of glutamine. Digests containing both sulfate and phosphate yielded the same results as those with phosphate alone.

Bicarbonate was very effective in accelerating the non-enzymatic desamidation of glutamine, but the pH of digests containing this salt was invariably higher than 8.0. At pH 8.4 ± 0.1, digests of glutamine with various salts at 0.2 M final concentration yielded the following amounts of
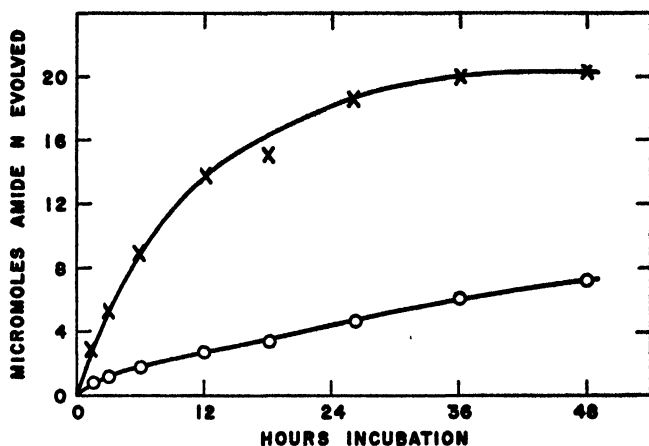
Fig. 1. Time course of non-enzymatic desamidation of glutamine. ○, no added phosphate; ×, with added phosphate. The mixtures consisted of 1 cc. of 0.025 M glutamine, 2 cc. of 0.08 M veronal acetate buffer at pH 8.0, and 1 cc. of either water or 0.8 M phosphate at pH 8.0. The initial and final pH of each mixture was 8.0 ± 0.05. Temperature 47°. Complete desamidation yields 22.5 μM of ammonia.
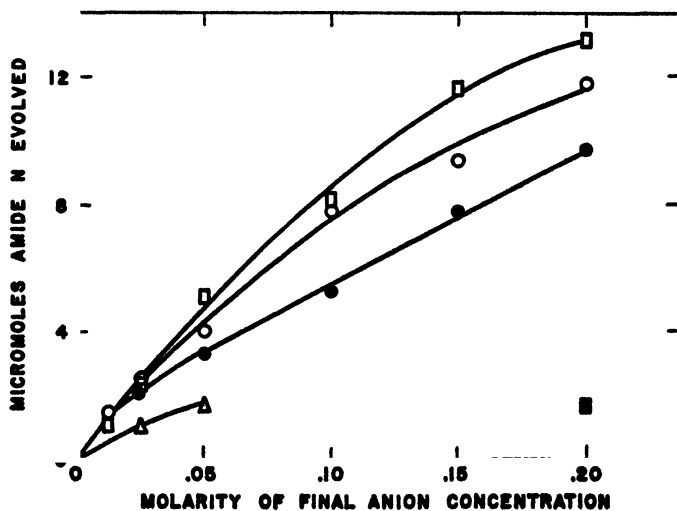
Fig. 2. Effect of various sodium salts on the non-enzymatic desamidation of glutamine. □, phosphate, ○, arsenate, ●, methyl arsenate, △, borate, ■, phosphite. The mixtures consisted of 1 cc. of 0.1 M glutamine, 2 cc. of 0.08 M veronal buffer at pH 8.0, and 1 cc. of water or salt solution at pH 8.0. The values were corrected for water blanks. The initial and final pH of each mixture was 8.0 ± 0.05. Incubation period, 8 hours at 37°.

amide nitrogen as micromoles of ammonia over the blanks: phosphate 18.1, arsenate 15.8, chloride 0, phosphite 1.7, and bicarbonate 12.1. The experimental conditions, other than those stated, were the same as those described in Fig. 2.

Effect of Phosphate Concentration at Different Concentrations of Gluta-mine—The data in Fig. 3 indicate that for a given concentration of phosphate the amount of desamidation is proportional to the concentration of glutamine up to about 30 per cent desamidation.
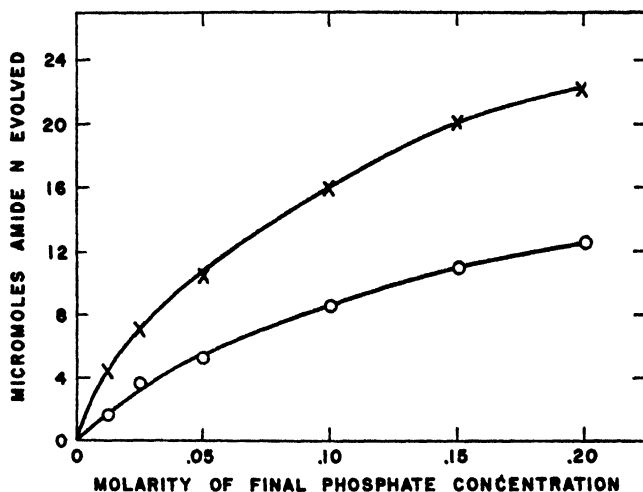
Fig. 3. Non-enzymatic desamidation of glutamine at two different concentrations. ×, 0.05 M; ○, 0.025 M glutamine. The mixtures consisted of 1 cc. of glutamine solution, 2 cc. of 0.08 M veronal buffer at pH 8.0, and 1 cc. of phosphate solution at pH 8.0. The initial and final pH of each mixture was 8.0 ± 0.05. The values were corrected for water blanks. Incubation period, 12 hours at 47°.

Effect of pH—The data in Fig. 4 demonstrate that a striking acceleration of the non-enzymatic desamidation of glutamine by phosphate occurs progressively with increasingly alkaline pH. In acid pH ranges, on the other hand, the desamidation is little affected by phosphate. The essential stability of glutamine at pH 4 to 7 in the absence of phosphate is revealed by Fig. 4. Sodium chloride added at the same ionic concentration as phosphate possessed no accelerating effect (Fig. 4).

Effect of Temperature—Digests consisting of 1 cc. of 0.1 M glutamine, 2 cc. of veronal buffer at pH 8.0, and 1 cc. of either water or 0.8 M phosphate were incubated at 47.4° and 37.0° for several hours. The initial and final pH of each digest was 8.0 ± 0.05, and the data were corrected for the small blanks in the absence of added phosphate. After 1.5 hours, the ammonia

liberated at the higher temperature in the presence of phosphate was 10.6 μM, and at the lower temperature 4.8 μM. After 3.25 hours, the corresponding values were 21.0 and 9.2 μM. On the basis of these data, the average temperature coefficient amounts to about 2.2 for a difference in temperature of 10.4°, or close to 2 for a difference of 10°.

Conversion of Glutamine to Pyrrolidonecarboxylic Acid—The non-enzymatic desamidation of glutamine is accompanied by ring closure to pyrrolidonecarboxylic acid (7, 8, 11). Phosphate, as well as bicarbonate and certain other salts, accelerates the reaction. We have noted that thi$_8$
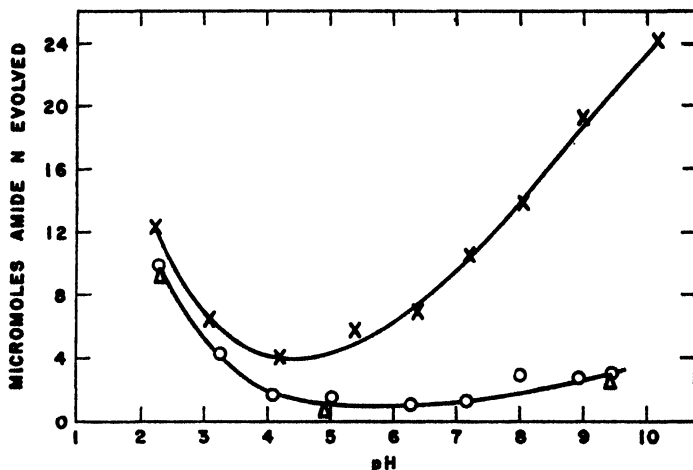
FIG. 4. Effect of pH on the non-enzymatic desamidation of glutamine. $\times$, 0.2 M final phosphate concentration; $\bigcirc$, no added salt; $\triangle$, sodium chloride added at equal ionic strength to phosphate. The mixtures consisted of 1 cc. of 0.05 M glutamine, 2 cc. of veronal buffer at different pH values, and either 1 cc. of water or 0.8 M phosphate. Incubation period, 12 hours at 37°.

acceleration by phosphate is not inhibited by pyrrolidonecarboxylic acid or glutamic acid added in a molar ratio of 400:1 of glutamine. When 1 cc. of ammonium glutamate, 2 cc. of veronal buffer at pH 8.0, and 1 cc. of 0.8 M phosphate at pH 8.0 were incubated for 12 hours at 37°, no appreciable loss of carboxyl nitrogen had occurred and thus no appreciable amount of pyrrolidonecarboxylic acid was formed. Under these conditions, therefore, glutamate is not converted to pyrrolidonecarboxylic acid even in the presence of phosphate.

Study of Chloroacetylglutamine—Mixtures of 1 cc. of neutralized 0.05 M chloroacetylglutamine (13), 2 cc. of 0.014 veronal buffer, and 1 cc. of either water or 0.8 M phosphate buffer at pH 8.0 were prepared and allowed to incubate at 47° for 4 and 8 hours. Under these conditions, and especially

in the presence of phosphate, glutamine yields an appreciable amount of its amide nitrogen as ammonia. With chloroacetylglutamine, however, no significant quantities of ammonia were noted, whether phosphate was or was not present. Chloroacetylation of the α-amino group of glutamine obviously blocks pyrrolidonecarboxylic acid formation.

Increased ammonia production from chloroacetylglutamine is noted when concentrated rat liver extracts treated with pyruvate (13) or phosphate (3) are used, but this may be due to a preliminary enzymatic hydrolysis at the peptide bond yielding free glutamine.

Effect of Various Anions on Enzymatic Desamidation of Glutamine—Phosphate, arsenate, and sulfate at 0.02 to 0.20 M concentration accelerate markedly the desamidation of glutamine in rat liver, kidney, spleen, and brain (3–6). Methyl arsenate and phosphate at concentrations lower than 0.02 M do not accelerate (4), but at higher concentrations do accelerate the enzymatic hydrolysis of the amide to practically the same extent as arsenate or phosphate. Since bicarbonate and borate accelerated the non-enzymatic desamidation of glutamine (see above), the effect of these salts at 0.10 M final concentration on the enzymatic desamidation of glutamine with rat liver extracts was studied. The digests consisted of 1 cc. of fresh aqueous rat liver extract equivalent to 330 mg. of tissue, 2 cc. of veronal buffer with and without the salts, and 1 cc. of either water or 0.014 M glutamine. The pH of the digests was 8.2 and the incubation period 1 hour at 37°. No measurable acceleration of desamidation of the glutamine by either borate or bicarbonate was noted.

Effect of pH and of Concentration on Accelerating Effect by Sulfate on Enzymatic Desamidation of Glutamine—The accelerating effect of sulfate on the enzymatic desamidation of glutamine is so unusual that a brief study was made of the reaction. In Fig. 5 is portrayed the effect of pH on this reaction, and it is noted that the pH of 8.0, when optimum desamidation occurs in the presence of sulfate, is practically the same as that of the optimum desamidation of glutamine in the absence of the added salt. Thus, the effect of sulfate in this respect is similar to that of phosphate or arsenate (3–6).

The effect of increasing amounts of sulfate on the enzymatic desamidation of glutamine is shown in Fig. 6. As in the case of added phosphate (3–6), the desamidation of glutamine in the presence of sulfate reaches a maximum value at a molar ratio of about 8 of salt to amide, and thereafter remains appreciably constant up to a final concentration of sulfate of 0.2 M.

Determinations of carboxyl nitrogen in digests of rat liver extracts with glutamine and sulfate revealed no evidence of appreciable pyrrolidonecarboxylic acid formation, for there was no apparent decrease in the car-
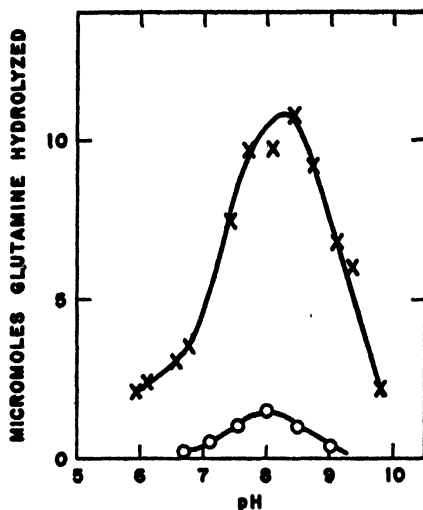
Fig. 5. Effect of pH on the enzymatic desamidation of glutamine by rat liver extracts in the presence and absence of added sodium sulfate. The digests consisted of 1 cc. of fresh aqueous extract equivalent to 330 mg. of tissue, 2 cc. of buffer with and without 0.4 M sodium sulfate, and 1 cc. of water or 0.014 M glutamine. $\times$, with SO_4; $\bigcirc$, without SO_4. Veronal buffers were used below pH 8.5, glycine-NaOH buffers above pH 8.5. Incubation period, 1 hour at 37°.
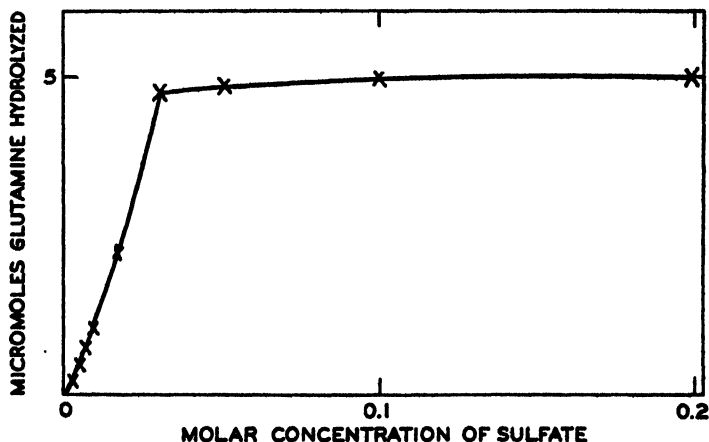
Fig. 6. Effect of concentration of sulfate on the enzymatic desamidation of glutamine. The digests consisted of 1 cc. of fresh aqueous rat liver extract equivalent to 330 mg. of tissue, 2 cc. of veronal acetate buffer at pH 8.0 with and without added sulfate, and 1 cc. of water or 0.014 M glutamine. Incubation period, 1 hour at 37°.

boxyl nitrogen titer after most of the substrate had been desamidated. Pyrrolidonecarboxylic acid itself was not attacked by rat liver, kidney, or brain extracts, whether added phosphate was present or not.

DISCUSSION

A summary of the effect of various anions on the respective enzymatic and non-enzymatic desamidation of glutamine is given in Table I. Phosphate, phosphite, arsenate, and methyl arsenate accelerate both the enzy-

TABLE I

Effect of Anions on Acceleration of Enzymatic and Non-Enzymatic Desamidation of Glutamine

Anion*	Increase in amide N hydrolyzed	
	Non-enzymatic†	Enzymatic‡
	μM	μM
Phosphate	13	8
Phosphite	1.5	6
Arsenate	12	7
Methyl arsenate	9.5	7
Borate	1.5	0
Bicarbonate	12	0
Sulfate	0	7.5
Nitrate	0	0
Acetate	0	0
Chloride	0	0
Pyruvate	0	6

* All sodium salts.

† The data were taken from Fig. 2. The experimental conditions and inactive anions are stated in the text.

‡ The data were obtained from (3–6, 14), Fig. 5, and the text of the present paper. Aqueous rat liver extracts were used. In all cases but pyruvate, the enzymatic desamidation refers to the activity of the widely distributed glutaminase I in rat tissues. Pyruvate is concerned only with the hepatic system, glutaminase II. The data on this system were obtained after 4 hours of incubation at pH 7.5.

matic and the non-enzymatic reactions. Nitrate, acetate, and chloride do not accelerate either the enzymatic or the non-enzymatic reactions. On the other hand, sulfate and pyruvate accelerate the enzymatic desamidation (by glutaminases I and II respectively) but not the non-enzymatic desamidation, while borate and bicarbonate accelerate the non-enzymatic but not the enzymatic desamidation.

The enzymatic and non-enzymatic desamidation of glutamine thus differ in at least three particulars: namely (a), the former yields glutamic acid and ammonia, whereas the latter reaction yields pyrrolidonecarboxylic

acid and ammonia; (b) the former is accelerated by sulfate and pyruvate but not by borate or bicarbonate, whereas the latter reaction is accelerated by borate and bicarbonate but not by sulfate or pyruvate; and (c) the enzymatic desamidation possesses an optimum at pH 8.0 in rat liver and kidney extracts, at pH 8.8 in rat brain extracts (3–6), and at pH 7.5 with dog kidney (2), whether added active anion is or is not present, whereas the non-enzymatic desamidation of glutamine possesses no optimum but simply increases progressively at either acid or alkaline reactions (Figs. 4 and 5). Moreover, the accelerating effect of phosphate on the non-enzymatic reaction increases with increasing pH and decreases with decreasing pH.

The complete enzymatic desamidation of glutamine is accomplished without detectable pyrrolidonecarboxylic acid formation. No enzyme is present, in any of the tissues studied, which is capable of hydrolyzing pyrrolidonecarboxylic acid to glutamic acid; and hence the primary action of tissue glutaminase is undoubtedly a simple hydrolysis at the amide bond to yield glutamic acid and ammonia. Since ammonium glutamate under the non-enzymatic conditions employed does not yield appreciable amounts of pyrrolidonecarboxylic acid, the non-enzymatic desamidation of glutamine is in effect the result of a simultaneous ring closure of the glutamine and not of a primary hydrolysis of the amide bond.

Bicarbonate, which accelerates the non-enzymatic desamidation of glutamine to an extent not much less than that of phosphate, has no influence on the enzymatic reaction under conditions whereby phosphate accelerates the reaction nearly to completion. It is true that the enzymatic reaction is very much faster than the non-enzymatic reaction, and, if bicarbonate had a very weak effect on the enzymatic desamidation, it might not have been noted in the relatively short period of incubation used (1 hour at 37°). On the other hand, sulfate accelerates the enzymatic desamidation of glutamine to a degree not much less than that of phosphate, but possesses no demonstrable effect on the non-enzymatic desamidation of glutamine under conditions whereby phosphate (and bicarbonate) considerably accelerates the reaction.

The data presented appear to indicate that the effect of the active anions on the enzymatic and on the non-enzymatic desamidation of glutamine may be fundamentally different. It is quite possible that the acceleration of non-enzymatic reaction by the active ions may be due to the formation of a highly labile intermediate complex of glutamine and anion in minute amounts. The presence of such a possible complex cannot be excluded in the course of the enzymatic reaction, but the enzyme itself, together with other tissue components in the crude extract, may produce conditions involving new specificities.

SUMMARY

1. The effect of various anions on the desamidation of glutamine in veronal-buffered solution has been studied and compared with the effect of the same ions on the enzymatic desamidation of this substrate in rat tissue aqueous extracts.

2. The non-enzymatic reaction which leads to the formation of pyrrolidonecarboxylic acid and ammonia is accelerated by phosphate, phosphite, arsenate, methyl arsenate, borate, and bicarbonate, and is not accelerated by sulfate, nitrate, acetate, chloride, or pyruvate at equivalent concentrations. Chloroacetylglutamine is not affected, whether phosphate is or is not present. The enzymatic reaction which leads to the formation of glutamic acid and ammonia is accelerated by phosphate, phosphite, arsenate, methyl arsenate, sulfate, and pyruvate, and is not accelerated by borate, bicarbonate, nitrate, acetate, or chloride at equivalent concentrations.

3. The desamidation of glutamine in tissue extracts occurs at an optimum at the same pH, whether accelerating salt has been added or not. The accelerating effect of phosphate on the non-enzymatic desamidation of glutamine increases with increasing pH, and decreases with decreasing pH.

4. The several differences between the phenomena of enzymatic and non-enzymatic desamidation reactions suggest that they occur by different mechanisms.

BIBLIOGRAPHY

1. Krebs, H. A., *Biochem. J.*, **29**, 1951 (1935).
2. Archibald, R. M., *J. Biol. Chem.*, **154**, 657 (1944).
3. Carter, C. E., and Greenstein, J. P., *J. Nat. Cancer Inst.*, **7**, 433 (1947).
4. Greenstein, J. P., and Leuthardt, F. M., *Arch. Biochem.*, **17**, 105 (1948).
5. Errera, M., *J. Biol. Chem.*, **178**, 483 (1949).
6. Errera, M., and Greenstein, J. P., *J. Biol. Chem.*, **178**, 495 (1949).
7. Chibnall, A. C., and Westall, R. G., *Biochem. J.*, **26**, 122 (1932).
8. Vickery, H. B., Pucher, G. W., Clark, H. E., Chibnall, A. C., and Westall, R. G., *Biochem. J.*, **29**, 2710 (1935).
9. Pucher, G. W., and Vickery, H. B., *Ind. and Eng. Chem., Anal. Ed.*, **12**, 27 (1940).
10. Harris, M. M., *J. Clin. Invest.*, **22**, 569 (1943).
11. Hamilton, P. B., *J. Biol. Chem.*, **158**, 375 (1945).
12. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).
13. Price, V. E., and Greenstein, J. P., *J. Nat. Cancer Inst.*, **7**, 275 (1947).
14. Greenstein, J. P., and Price, V. E., *J. Biol. Chem.*, **178**, 695 (1949).
15. Mylon, E., and Heller, J. H., *Am. J. Physiol.*, **154**, 542 (1948).

BIOPHYSICAL STUDIES OF BLOOD PLASMA PROTEINS

XII. ELECTROPHORETIC STUDIES OF THE BLOOD SERUM PROTEINS OF SOME LOWER ANIMALS*

BY H. F. DEUTSCH AND W. H. McSHAN

(From the Departments of Physiological Chemistry, Chemistry, and Zoology, University of Wisconsin, Madison)

The blood serum (1) and plasma (2) proteins of various relatively easily accessible animals have been studied previously by the electrophoretic technique. The serum proteins of each species were found to give characteristic species variations in mobility, amount, and number of protein components.

An electrophoretic study of the blood serum proteins of various animals of relatively lower phylogenetic order has revealed that the same species specificity of the plasma proteins that exists among the higher animals likewise obtains among the lower forms.

EXPERIMENTAL

With the exception of the frog, turtle, horseshoe crab, and snail, blood samples were obtained from all species by heart puncture. Samples from the frog and turtle were collected from a severed artery. The snail and horseshoe crab were bled from a main circulatory sinus. The blood from as many animals of a given species as it was possible to obtain was pooled and the serum recovered by the usual methods. In some cases only a single animal of a given species was available. An analysis of total serum proteins (serum N $\times$ 6.25) was carried out by using a small aliquot of each sample. The remaining serum was dialyzed for 36 to 72 hours at 1-2° against several changes of a pH 8.6, sodium diethylbarbiturate buffer of ionic strength, 0.1. The samples were then diluted with buffer of this composition to give solutions of 2 to 3 per cent protein concentration (if more concentrated) and subjected to electrophoretic analysis. A diagonal knife-edged schlieren photographic method was used to record the position of the moving boundaries as a function of time. A constant potential gradient of approximately 6 volts per cm. was used for the electrophoresis experiment. The duration of any given experiment depended on the electrophoretic resolution obtained.

No attempt has been made to identify the protein components of the serums studied as being albumin or one of the several globulins; rather the electrophoretic areas were merely assigned numbers in order of increasing electrophoretic mobility. Percentage composition and mobilities for the areas were calculated from the descending electrophoretic pattern. The position of the electrophoretic salt anomaly on the descending side is indicated by ϵ in the diagrams, while the corresponding boundary on the ascending side is designated as δ.

Results

Data concerning the species studied, the number of animals used in each case, serum protein levels, and the percentages of the various proteins and their electrophoretic mobilities are given in Table I. For the purposes of greater clarity, the electrophoretic patterns of each species will be discussed individually and in phylogenetic sequence.

Snakes—Blood serums from five species of snakes were studied. A species specificity as revealed by the electrophoretic analysis of these proteins is apparent. The serum proteins of the water-snake (Fig. 1), milk-snake (Fig. 2), and moccasin (Fig. 3) give unique electrophoretic patterns. The water-snake serum proteins contain large amounts of low mobility components. In mammalian serum such proteins are characteristically globulin in nature. Even two such closely related species as the diamond back and timber rattlesnakes (Figs. 4 and 5 respectively) give patterns which are easily distinguishable. The diamond back rattlesnake shows ten components and Component 3 appears to consist of at least three components. The diagram of the timber rattlesnake serum indicates the presence of at least nine distinct components, with a markedly different concentration distribution as compared to the components of a corresponding number in the diamond back rattlesnake. All of the snake serum protein diagrams have the common property of possessing a relatively strong anomaly in the region of Component 2 or 3 of the descending electrophoretic pattern.

Turtles—Two species of turtles were studied. Electrophoretic patterns of the serum proteins of the soft-shelled and snapping turtles are shown in Figs. 6 and 7, respectively. These diagrams reveal complete lack of similarity. The snapping turtle's serum proteins consist largely of molecules having relatively low mobilities in contrast to those of the soft-shelled turtle. A strong anomaly shown in the region of Component 2 of the soft-shelled turtle is a further distinguishing feature of these patterns. The ascending limb of the electrophoretic diagram of the serum proteins of both these species reveals that these systems are more complex than the analytical data of Table I would suggest.

Bullfrog—The electrophoretic pattern of the serum proteins of this amphibian is shown in Fig. 8. Nine components whose mobility and percentage distribution appear to be characteristic for this species were analyzed. The complexity of this pattern, however, suggests that many more proteins are present. Thus the ascending diagram shows that Component 2 is definitely a composite of several proteins.

Perch—A relatively complex electrophoretic pattern is shown by the perch serum proteins in Fig. 9. Twelve components were analyzed. The poor resolution of many of them tends to make the analytical values of Table I somewhat arbitrary.

Wall-Eyed Pike—The electrophoretic pattern of this member of the family Percidae is seen in Fig. 10. At least eleven components are present. They show relatively good resolution on the ascending side. These proteins do not show much electrophoretic similarity to the serum proteins of the closely related perch.

Rock Bass—Fig. 11 shows the electrophoretic data for a serum pool of this species. Nine relatively well defined components are present.

Catfish—Four species of the family Ameiuridae were studied. Two of these members, the channel and white catfish, are classified as belonging to the same genus and species. They are, however, distinguishable morphologically and the electrophoretic patterns of their serum proteins do show considerable difference. These patterns for the channel and the white catfish are shown in Figs. 12 and 13, respectively. Eight components are seen in the white catfish serum, Components 7 and 8 comprising the major portions of the protein. The channel catfish serum proteins likewise show eight components with a considerable similarity in their mobilities. The amounts of the individual proteins, however, vary considerably; thus Component 7 of the white catfish is the major serum component, while in the case of the channel catfish it is a relatively small component. Because of this marked variation the electrophoretic diagrams of the serum proteins of these two species are readily distinguished.

The serum protein diagram of the eel catfish (Fig. 14) shows further variations from the electrophoretic diagrams of the serum protein of the channel and the white catfish. However, certain similarities to the diagram of the white catfish are evident. Eight components are present, but the amounts of proteins present in these areas vary considerably from those of the other catfish, as shown by the data of Table I. The serum of a fourth member of the family Ameiuridae, the bullhead, gives the electrophoretic pattern shown in Fig. 15. Again eight components are present, but with a great deal of variation from the other species of this family. The major portion of the protein is carried by Components 7 and 8, which are closely related in electrophoretic mobility and hence

TABLE I

Electrophoretic Analyses of Serum Proteins

For each component, the values for the first reading are given in per cent; for the second reading in mobility $\times 10^5$ sq. cm. volt^{-1} sec.$^{-1}$.

Family and species	No. of animals	Serum proteins (N × 6.25) per cent	1	2	3	4	5	6	7	8	9	10	11	12
Colubridae														
Water-snake, *Natrix sipedon*	2	7.6	16.6	17.9	30.7	5.0	10.3	3.5	6.6	9.4				
			3.8	4.1	5.7	6.9	7.6	8.8	10.0	11.7				
Milk-snake, *Lampropeltis triangulum*	1	6.8	1.3	5.7	16.4	11.6	5.9	5.8	11.9	23.8	17.6			
			1.4	2.0	2.6	3.4	3.8	4.4	5.1	6.5	7.9			
Crotalidae (Viperidae)														
Water-moccasin, *Agkistrodon piscivorus*	1	5.5	2.5	15.8	18.8	13.5	5.4	5.3	11.3	6.4	5.4	15.6		
			1.0	1.8	2.8	3.9	4.5	5.0	5.7	6.4	6.8	7.4		
Rattlesnake, diamond back, *Crotalus atrox*	2	5.1	4.4	5.9	6.0	21.0	8.7	3.9	8.7	4.0	4.6	32.8		
			1.7	2.6	3.2	3.7	4.3	4.7	5.3	5.8	6.3	7.3		
Rattlesnake, timber, *Crotalus horridus*	1	6.5	3.8	3.5	19.7	9.4	8.8	13.3	10.3	28.4	2.8			
			3.4	4.7	6.2	7.4	8.7	8.0	11.0	12.4	14.0			
Ranidae														
Bull-frog, *Rana catesbiana*	5	2.5	8.4	7.0	5.5	5.8	12.0	22.4	16.0	22.4	0.5			
			1.5	2.3	3.1	3.7	4.3	4.9	5.6	6.5	7.2			
Trionychidae														
Turtle, soft-shelled, *Amyda spinifera*	3	5.0	1.9	15.5	11.6	17.6	51.8	1.6						
			1.4	2.5	3.5	4.4	5.8	6.9						
Chelydridae														
Turtle, snapping, *Chelydra serpentina*	8	5.1	5.3	20.5	31.5	16.6	7.5	16.2	2.4					
			1.1	1.8	3.8	3.6	4.5	5.3	6.1					
Cyprinidae														
Carp, *Cyprinus carpio*	7	3.2	4.1	8.2	14.3	15.0	21.5	36.9						
			2.3	3.7	4.7	5.7	7.1	8.6						

	N													
Ameiuridae														
Catfish, channel, *Ictalurus lacustris*	25	4.3	2.9	3.8	3.2	6.6	6.6	11.2	20.6	45.1				
			1.3	1.8	2.3	3.0	3.9	4.7	5.7	6.5				
Catfish, eel, willow cat, *Ictalurus anguilla*	4	4.5	6.2	7.8	4.3	5.1	14.1	32.4	4.2	25.9				
			1.7	2.3	3.1	3.7	4.4	5.6	6.7	7.7				
Catfish, white, *Ictalarus lacustris*	3	3.3	1.9	5.2	4.5	7.4	5.3	10.8	36.0	28.9				
			1.1	1.9	2.7	3.2	3.9	4.7	5.6	6.5				
Bullhead, *Ameiurus* sp.	25	3.8	0.6	4.7	8.5	15.8	7.3	9.3	30.6	23.2				
			1.8	3.0	4.0	5.4	6.8	7.6	8.7	10.0				
Catostomidae														
Sucker, *Catostomus commersonii*	8	5.1	1.4	13.6	14.2	12.1	18.7	7.6	31.8	0.6				
			1.4	2.1	3.0	3.8	4.9	5.5	6.4	7.4				
Buffalo-fish, *Ictiobus bubalus*	1	4.5	2.5	25.0	10.2	9.7	17.8	5.5	5.5	23.8				
			1.4	2.5	3.1	3.9	4.7	5.4	6.2	6.9				
Acipenseridae														
Sturgeon 1, rock, *Acipenser* sp.	1	3.1	22.6	8.5	10.4	5.6	5.3	18.3	11.5	8.9	7.4	1.5		
			1.7	2.3	3.0	3.6	4.3	5.0	5.6	6.2	6.9	7.4		
Sturgeon 2, rock, *Acipenser* sp.	1	2.5	17.9	18.2	7.6	2.9	6.0	15.0	6.5	25.1	0.8			
			1.5	2.2	2.7	3.2	3.7	4.1	4.5	5.2	5.9			
Percidae														
Wall-eyed pike, *Stizostedion vitreum*	8	5.8	1.1	4.1	5.6	3.0	4.0	13.0	4.7	13.2	9.5	8.0		
			2.7	3.8	4.7	5.4	5.9	6.7	7.6	7.8	10.0	11.0		
Perch, yellow, *Perca flavescens*	25	4.5	1.8	2.6	7.0	4.6	16.6	8.8	4.4	5.2	7.0	8.0	11.0	33.8
			2.5	2.9	3.5	4.4	5.0	5.8	6.5	7.2	8.0	10.0	11.0	11.9
Salmonidae														
Trout, rainbow, *Salmo gairdnerii irideus*	6	5.1	1.2	1.6	3.4	4.0	4.0	6.7	45.6	2.8				
			1.3	2.0	2.7	3.1	4.2	5.1	6.3	7.4				
Trout, lake, *Cristivomer namaycush*	8	5.0	1.4	3.8	7.9	6.5	8.2	12.8	58.4	1.0				
			1.3	2.2	2.7	3.4	4.2	4.9	6.5	7.5				
Trout, German brown, *Salmo trutta fario*	4	4.0	1.1	3.2	7.2	15.0	20.2	7.6	11.0	16.9	9.6	7.4	0.8	1.2
			1.4	1.9	2.5	3.2	4.2	5.1	5.5	6.0	6.5	7.4	8.0	10.8

TABLE I—*Concluded*

Family and species	No. of animals	Serum proteins (N × 6.25)	1	2	3	4	5	6	7	8	9	10	11	12
		per cent												
Salmonidae (Coregonidae)														
Whitefish, *Coregonus clupeaformis*	25	5.8	0.5	7.5	11.7	12.7	8.1	14.6	12.1	17.6	14.7	0.5		
			1.3	2.7	3.0	3.5	4.1	4.8	5.7	5.9	7.0	7.7		
Herring, lake, *Leucichthys artedi arcturus*	25	5.0	1.7	12.4	7.1	7.5	5.4	9.9	4.4	6.6	17.0	26.5	1.2	
			1.4	2.4	3.2	3.8	4.3	4.8	5.3	6.1	6.8	7.7	8.5	
Esocidae														
Pike, northern, *Esox lucius*	2	3.3	7.8	12.2	6.3	7.8	48.2	13.5	4.2					
			1.9	2.8	3.7	4.3	5.7	8.0	8.7					
Centrarchidae														
Bass, rock, *Ambloplites rupestris*	20	4.0	1.3	5.7	2.9	9.3	21.2	17.3	10.7	27.9	0.7			
			2.3	3.0	3.6	4.3	5.4	6.1	7.0	7.9	8.8			
Polygyridae														
Land snail, *Polygyra*	25	2.2	2.7	2.0	4.8	7.0	13.6	62.3	7.6					
			2.4	3.5	4.7	6.2	7.1	7.9	9.5					
Xiphosuridae														
Horseshoe crab, *Limulus polyphemus*	2	7.5	4.0	7.5	10.7	24.5	53.3							
			2.0	4.5	5.1	6.1	6.7							

show poor resolution. As will be revealed later, the members of this family do, however, show greater serum protein similarities as measured by the electrophoretic technique than do the trout members of the family Salmonidae.

FIG. 1. Electrophoretic diagram of water-snake blood serum proteins. Duration of experiment 10,800 seconds.

FIG. 2. Electrophoretic diagram of milk-snake blood serum proteins. Duration of experiment 9000 seconds.

FIG. 3. Electrophoretic diagram of moccasin snake blood serum proteins. Duration of experiment 10,800 seconds.

Carp—The electrophoretic pattern for pooled carp serum is shown in Fig. 16. It does not agree well with the pattern previously reported by Deutsch and Goodloe (2) for this species, as found in a barbiturate-citrate buffer of pH 8.6. The major portions of these proteins have relatively high mobilities, usually characteristic of serum albumins. This agrees with the high albumin to globulin ratio for carp serum, as determined chemically by Field *et al.* (3). The protein level of the pooled carp serum

used in these studies falls in the lower range of the values found by the above investigators.

FIG. 4. Electrophoretic diagram of diamond back rattlesnake blood serum proteins. Duration of experiment 10,800 seconds.

FIG. 5. Electrophoretic diagram of timber rattlesnake blood serum proteins. Duration of experiment 10,800 seconds.

FIG. 6. Electrophoretic diagram of soft-shelled turtle blood serum proteins. Duration of experiment 10,800 seconds.

FIG. 7. Electrophoretic diagram of snapping turtle blood serum proteins. Duration of experiment 10,800 seconds.

Sucker—The serum proteins of this species give the electrophoretic diagram shown in Fig. 17. The pattern resembles those of the carp and the whitefish in that they are relatively closely grouped in regard to their

electrophoretic mobilities, and hence show poor resolution. Components 3 and 5 give evidence of being composite in character.
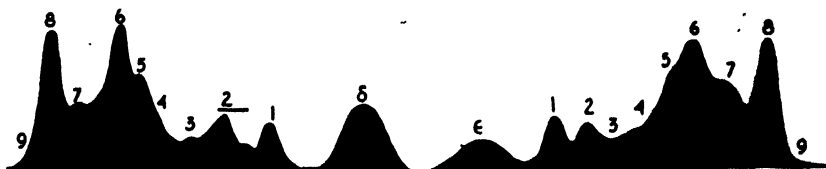
FIG. 8. Electrophoretic diagram of bullfrog blood serum proteins. Duration of experiment 10,800 seconds.
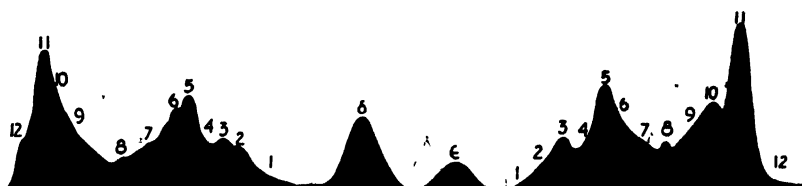
FIG. 9. Electrophoretic diagram of perch blood serum proteins. Duration of experiment 7380 seconds.
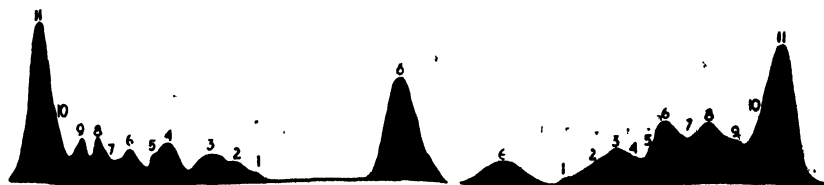
FIG. 10. Electrophoretic diagram of wall-eyed pike blood serum proteins. Duration of experiment 10,800 seconds.

FIG. 11. Electrophoretic diagram of rock bass blood serum proteins. Duration of experiment 8880 seconds.

Buffalo-Fish—In Fig. 18 is shown the electrophoretic diagram for the serum proteins of this species. The individual components, eight in number, are grouped into three more or less distinct regions of approximately equal size.

Whitefish—At least ten electrophoretic serum protein components are

shown by this species (Fig. 19). The individual proteins are poorly resolved, which makes difficult their analyses for percentage composition and mobility.
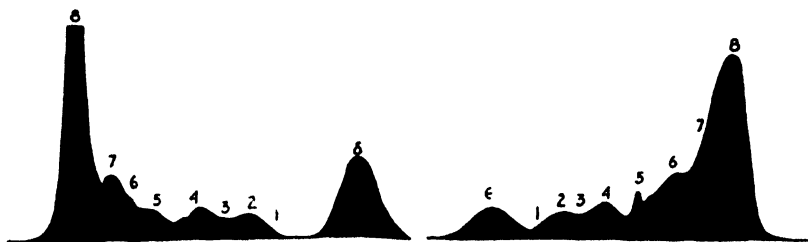
FIG. 12. Electrophoretic diagram of channel catfish blood serum proteins. Duration of experiment 10,800 seconds.
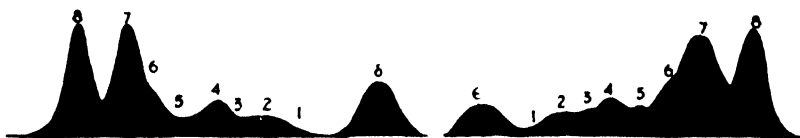
FIG. 13. Electrophoretic diagram of white catfish blood serum proteins. Duration of experiment 10,800 seconds.

FIG. 14. Electrophoretic diagram of eel catfish blood serum proteins. Duration of experiment 10,800 seconds.

FIG. 15. Electrophoretic diagram of bullhead blood serum proteins. Duration of experiment 10,800 seconds.

Herring—An electrophoretic diagram of the serum proteins of this species is shown in Fig. 20. Eleven components showing wide variation in mobility are present.

Trout—Three trout species were available. The German brown and

rainbow trout were hatchery-reared, while the lake trout were obtained from their native habitat (Lake Michigan). The electrophoretic pattern for the rainbow trout shown in Fig. 21 reveals the presence of eight components. Of these Components 5 and 7 make up the major portions of
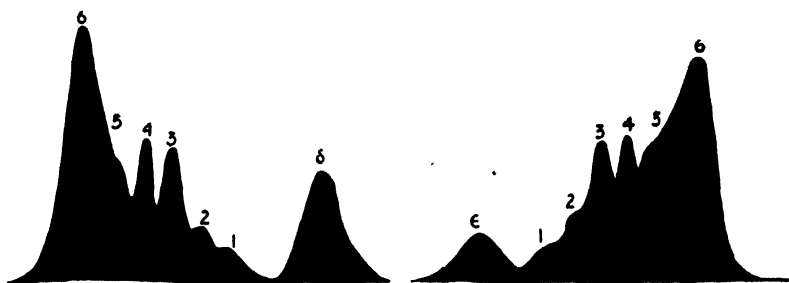
Fig. 16. Electrophoretic diagram of carp blood serum proteins. Duration of experiment 10,080 seconds.
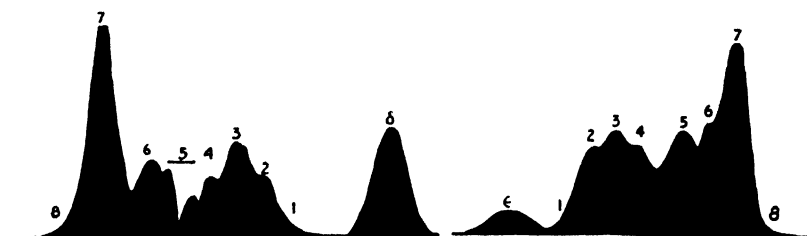
Fig. 17. Electrophoretic diagram of sucker blood serum proteins. Duration of experiment 10,860 seconds.
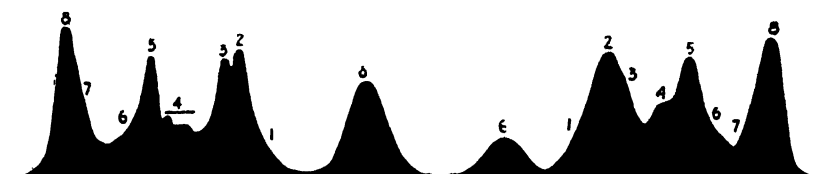
Fig. 18. Electrophoretic diagram of buffalo-fish blood serum proteins. Duration of experiment 10,800 seconds.

the total protein. This serum is characterized by the relatively high electrophoretic mobility of the components. The electrophoretic pattern of the serum proteins of the German brown trout is shown in Fig. 22. A very complex pattern showing at least eleven components appears. No particular relation to the serum proteins of the closely related rainbow

trout reared under identical conditions is revealed.   A characteristic elec-
trophoretic pattern is likewise shown by the serum proteins of the lake
trout (Fig. 23).   Eight distinct areas are revealed.   As seen in Table I,
Component 7 makes up approximately 60 per cent of the serum proteins.
It agrees rather closely in electrophoretic mobility with Component 7
of rainbow trout serum.   In any event, the serum proteins of these three
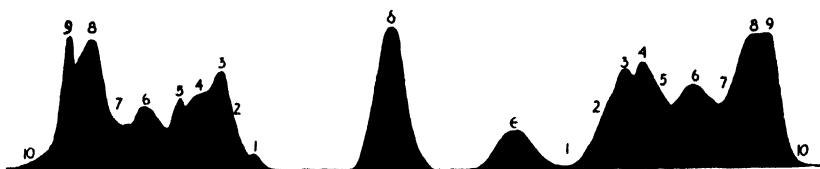
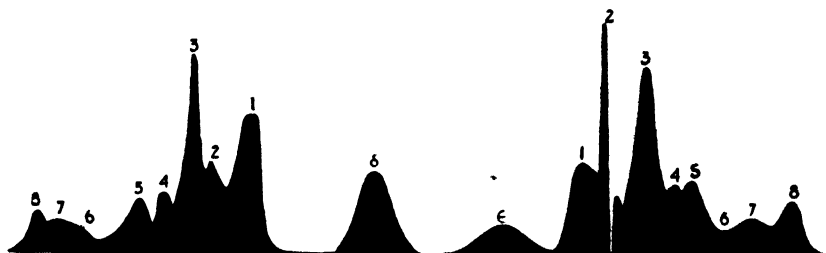

FIG. 19. Electrophoretic diagram of whitefish blood serum proteins.   Duration of
experiment 11,280 seconds.
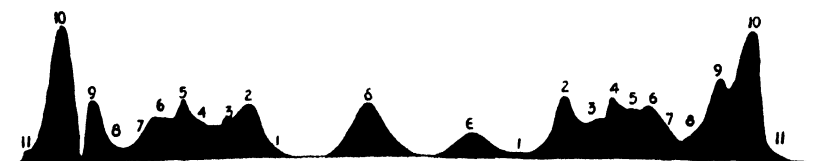
FIG. 20. Electrophoretic diagram of herring blood serum proteins.   Duration of
experiment 9780 seconds.
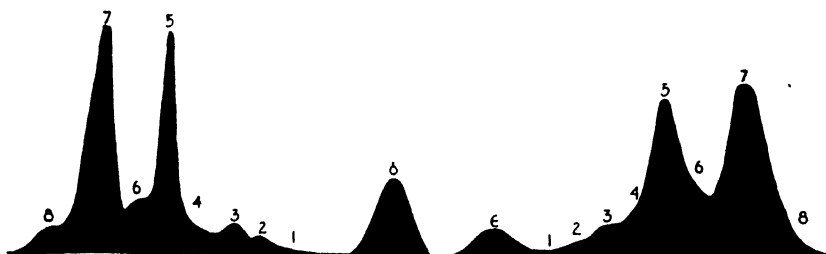
FIG. 21. Electrophoretic diagram of rainbow trout blood serum proteins.   Dura-
tion of experiment 10,800 seconds.

closely related fish species show marked differences as revealed by their
electrophoretic patterns.

Northern Pike—The electrophoretic diagram for the serum proteins
of this species is shown in Fig. 24.   Seven components were analyzed.
They show poor resolution and bear no resemblance to the proteins of the
wall-eyed pike.

Sturgeon—Two samples of rock sturgeon blood were obtained.   The

serums from these samples were analyzed individually; serums from Sturgeons 1 and 2 are shown in Figs. 25 and 26 respectively. Considerable variation is apparent. The sample from Sturgeon 1 showed evidences

FIG. 22. Electrophoretic diagram of German brown trout blood serum proteins. Duration of experiment 10,800 seconds.
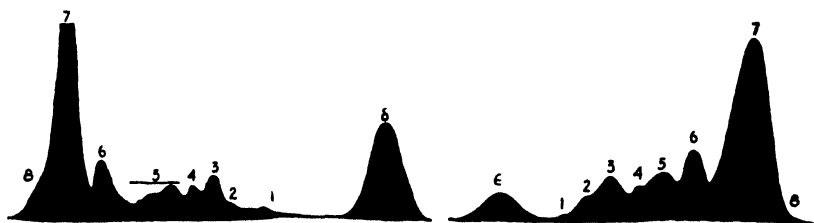
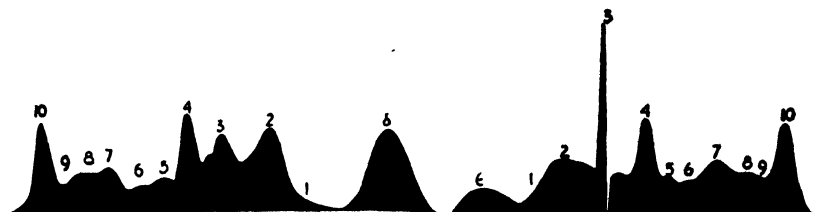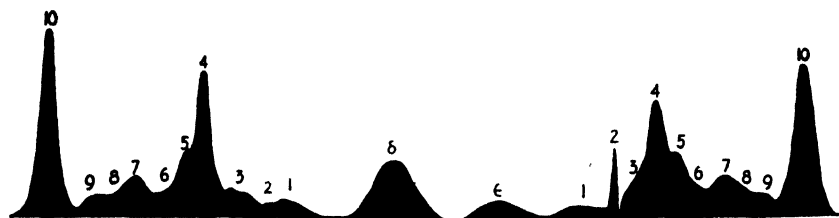FIG. 23. Electrophoretic diagram of lake trout blood serum proteins. Duration of experiment 10,800 seconds.
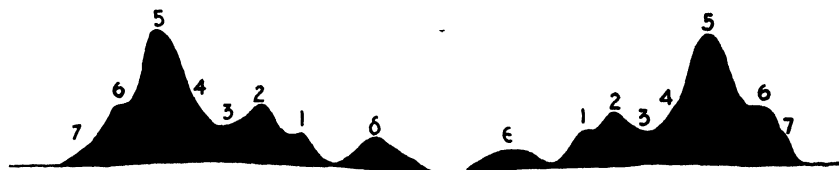
FIG. 24. Electrophoretic diagram of northern pike blood serum proteins. Duration of experiment 7200 seconds.
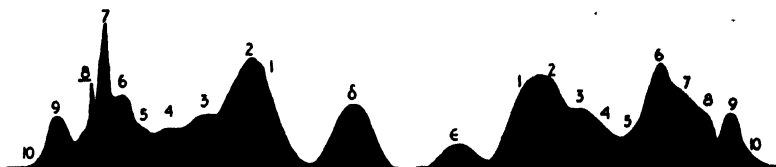
FIG. 25. Electrophoretic diagram of Sturgeon 1 blood serum proteins. Duration of experiment 10,800 seconds.

of at least ten components, while only nine were apparent in that of Sturgeon 2. The essential difference in the electrophoretic pattern of the two serums is an enhanced amount of the faster migrating proteins in the

serum from Sturgeon 2.   Proteins in this area of the electrophoretic pat-
terns of higher animals are generally albumin in nature.   It would thus
appear that the sample of Sturgeon 2 (Fig. 26) might contain a greater
amount of albumin.   A further difference is the marked anomaly in serums
from Sturgeon 2 in Component 2 of the descending side.   This anomaly
is usually representative of lipoprotein components in serum of higher
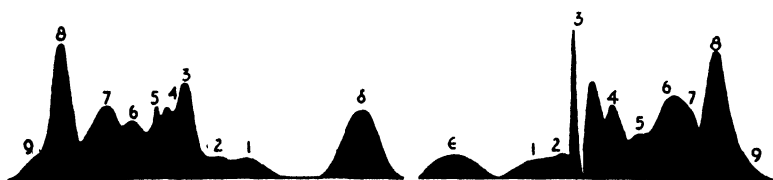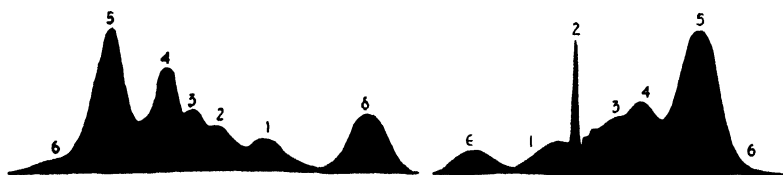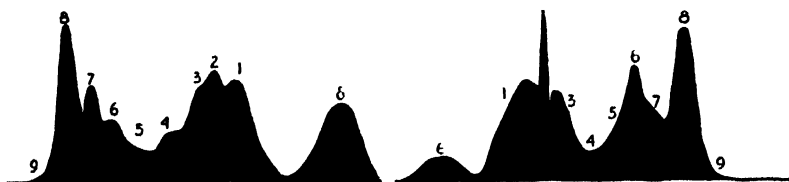

Fig. 26. Electrophoretic diagram of Sturgeon 2 blood serum proteins.   Duration
of experiment 13,500 seconds
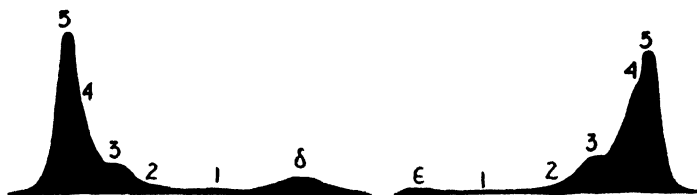
Fig. 27. Electrophoretic diagram of horseshoe crab blood serum proteins   Dura-
tion of experiment 7200 seconds.
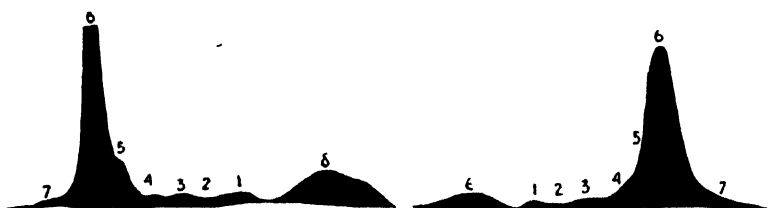
Fig. 28. Electrophoretic diagram of snail blood serum proteins.   Duration of
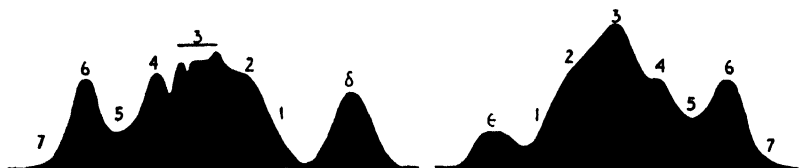experiment 11,400 seconds.

animals and the designation of β-globulins is usually given to the proteins
in this region of the pattern.

Horseshoe Crab—The blood proteins of this species and the snail (*Poly-
gyria*) depart from those of the other animals studied in that the respira-
tory proteins do not exist in cellular form.   It is generally regarded that
the blood serum protein of *Limulus* (horseshoe crab) is essentially hemo-
cyanin.   Previous electrophoretic analyses of *Limulus hemocyanin* over a

pH range of 5.0 to 10.5 by Svedberg and Pedersen (4) suggested the presence of only one component. The electrophoretic pattern shown in Fig. 27 reveals at least five areas of which Components 1 and 2 exist in very low concentration. The major portion of these blood proteins is indicated by the poorly resolved Components 4 and 5. It is likely that these components as well as Component 3 are hemocyanins.

Snail—The proteins of the blood of this species are largely carried by Component 6. In addition, it can be seen from Fig. 28 that six other small components are present. It is not known whether the main electrophoretic peaks represent respiratory pigment or pigments of this blood.

DISCUSSION

All of the serum protein systems studied in this work show electrophoretic patterns which appear to be characteristic of the species. In many cases even closely related species such as members of the family Salmonidae (trout), Ameiuridae (catfish), and Reptilia (snakes) show extreme and easily recognizable variations. The variation among individual species encountered in this work was much greater than that seen between man and monkey serum proteins and between those of a group of birds (2).

The blood serum samples analyzed often consisted of collections from only one or two members of a given species. Individual serum protein fluctuations, as evaluated by the electrophoretic technique, are known to exist, particularly as a response to various pathologies (5, 6). However, the individual variations in apparently normal animals are relatively small. The differences of species encountered in this work on the serum of single individuals or of two or three animals appear to be far out of the range of such variations. However, the analysis of two samples of rock sturgeon blood serum did reveal rather startling differences.

A general characteristic of the serum proteins studied in this work is the relatively low amount or even absence of a component comparable to the γ-globulin in the serum of mammals. The component of like mobility in the serum of the lower animals, designated Component 1 in Figs. 1 to 28, is generally present in much lower amounts. Furthermore, this component generally has a higher mobility as compared to the value usually given for the normal mammalian serum γ-globulins (7–9).

The well known so called β anomaly of mammalian serum again appears in the electrophoretic patterns for the serums of many of the lower animals. The actual mobilities of this component are not greatly different from the analogous mammalian serum proteins.

Only a limited amount of information is available on the blood serum proteins of lower animals (3, 10–13). The great complexity of the serum

proteins which is indicated by the electrophoretic method suggests the necessity of much further detailed chemical study. The present study has sufficed merely to show the complexity of these protein systems and to indicate our almost complete lack of any specific knowledge as regards the nature of the individual protein components.

SUMMARY

The blood serums of twenty-eight lower animals, including the syneretic fluid of the bloods of two invertebrates, were subjected to electrophoretic analysis. The same species specificity as shown by higher animals exists at the lower phylogenetic levels.

BIBLIOGRAPHY

1. Moore, D. H., *J. Biol. Chem.*, **161**, 21 (1945).
2. Deutsch, H. F., and Goodloe, M. B., *J. Biol. Chem.*, **161**, 1 (1945).
3. Field, J. B., Elvehjem, C. A., and Juday, C., *J. Biol. Chem.*, **148**, 261 (1943).
4. Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford (1940).
5. Stern, K. G., and Reiner, M., *Yale J. Biol. and Med.*, **19**, 67 (1946).
6. Luetscher, J. A., *Physiol. Rev.*, **27**, 621 (1947).
7. Nichol, J. C., and Deutsch, H. F., *J. Am. Chem. Soc.*, **70**, 80 (1948).
8. Hess, E. L., and Deutsch, H. F., *J. Am. Chem. Soc.*, **70**, 84 (1948).
9. Deutsch, H. F., and Nichol, J. C., *J. Biol. Chem.*, **176**, 797 (1948).
10. Lepkovsky, S., *J. Biol. Chem.*, **85**, 667 (1929–30).
11. Vars, H. M., *J. Biol. Chem.*, **105**, 135 (1934).
12. Roche, J., Derrien, Y., and Chouaiech, M. S., *Ann. inst. océanog.*, **20**, 97 (1940); *Chem. Abstr.*, **35**, 6324 (1941).
13. Joy, and Dastugue, G., *Chem. Abstr.*, **40**, 5498 (1946).

THE CHEMISTRY OF MELANIN

V. OXIDATION OF DIHYDROXYPHENYLALANINE BY TYROSINASE[*]

By HOWARD S. MASON and CHARLES I. WRIGHT

(From the Clinical Investigations Section, Division of Industrial Hygiene, and the Experimental Biology and Medicine Institute, National Institutes of Health, United States Public Health Service, Bethesda, Maryland)

The sequence of reactions which occurs when 3,4-dihydroxyphenylalanine is oxidized in the presence of tyrosinase is now considered to be as in the accompanying diagram (1, 2). This process has been reported to require from 3.2 to 4.12 atoms of oxygen per molecule of dihydroxyphenylalanine (3–5). Detection of the carbon dioxide which should be evolved during this reaction sequence has not, to our knowledge, been reported. In the present study the oxygen consumed under varying conditions of pH, enzyme concentration, and buffer salts has been determined; carbon dioxide evolved under corresponding conditions but at a fixed pH, 5.1, was also measured. The results were found to support and extend the above concept of melanogenesis.

EXPERIMENTAL

The measurements of gaseous exchange were made with Barcroft type differential manometers fitted with side arm flasks of approximately 18 ml. capacity. The reagents in each flask included 0.2 ml. of 10 per cent KOH or buffer in the central well. The fluid volume was brought to 3.0 ml. by employing 2.3 ml. of buffer containing 0.65 mg. of dihydroxyphenylalanine (3.3×10^{-6} mole) and 0.5 ml. of tyrosinase in distilled water (side arm). Further details of the manometric technique have been given in a previous paper (6). Carbon dioxide was determined by difference in the apparent oxygen consumption in the presence and absence of KOH, properly corrected by the CO_2 factor. By experiment it was shown that no carbon dioxide was retained as bicarbonate at pH 5.1.

The tyrosinase employed in this study was purchased from the Treemond Company[1] and was labeled to contain 3500 catecholase units per ml. (8). 3,4-Dihydroxyphenyl-L-alanine and 3,4-dihydroxyphenyl-DL-alanine were obtained from Hoffmann-La Roche, Inc. They melted at

---

[*] For the preceding paper in this series, see Mason, H. S., Kahler, H., MacCardle, R. C., and Dalton, A. J., *Proc. Soc. Exp. Biol. and Med.*, **66**, 421 (1947).

[1] The enzyme was prepared according to the procedure of Mallette *et al.* (7) from the common mushroom (personal communication).

272–278° and 270–274° (corrected) respectively. Unless otherwise noted, the raceme was employed as substrate in the experiments described below. The phosphate-citrate buffers referred to had the following compositions: at pH 6.6, 0.273 M disodium phosphate and 0.027 M citric acid; at pH 7.4, 0.305 M disodium phosphate and 0.012 M citric acid. Other buffers were prepared from 0.2 M phosphate, citrate, and acetate solutions of the desired pH by direct dilution. Measurement showed that dilution from 0.2 to 0.1 M caused a pH increase in the phosphate buffers of less than

0.1 unit, and in the citrate buffer, 0.2 unit. Dilution of from 0.2 to 0.01 M caused a pH increase of 0.2 to 0.3 unit in the phosphate buffers, and 0.4 unit in the citrate buffer. The acetate buffer was not affected by dilution in the range of pH and concentration utilized. The effect of dilution upon hydrogen ion concentration must be taken into account in interpreting Figs. 2, 5, 7, and 8. Buffer concentrations are given below in terms of final values in the reaction vessels.

Results

Fig. 1 shows the results obtained when 0.65 mg. of dihydroxyphenylalanine was oxidized in the presence of increasing amounts of enzyme at pH 7.4 and 6.6. In 0.164 M phosphate buffer (pH 7.4) concentrations

of enzyme between 22 and 175 units catalyzed the consumption of oxygen to 170 μl. in 240 minutes; with 11 units the oxygen consumption dropped to 132 μl. In phosphate-citrate buffer at the same pH, increasing the tyrosinase concentration from 11 to 44 units increased the oxygen consumed in 240 minutes from 130 to 170 μl.; further increase of enzyme
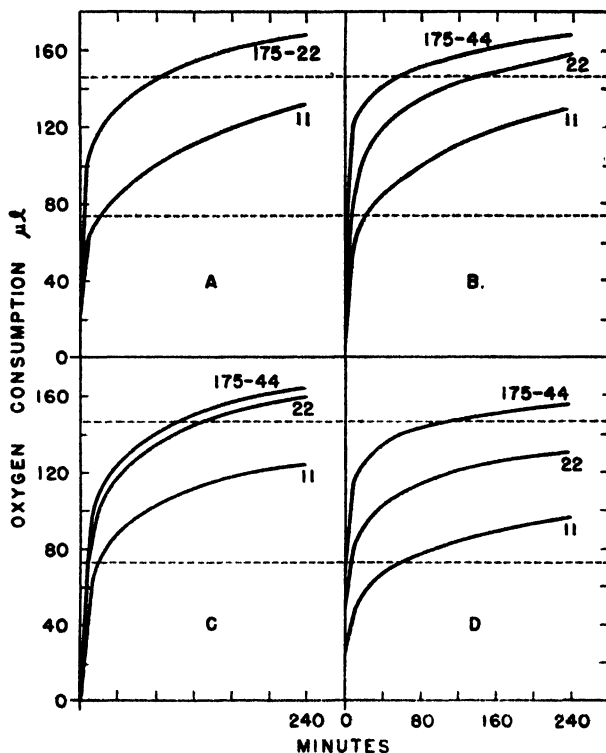
FIG. 1. The oxygen consumed during the enzymic oxidation of 0.65 mg. of dihydroxyphenyl-DL-alanine with increasing quantities of tyrosinase in (A) 0.164 M phosphate buffer, pH 7.4; (B) phosphate-citrate, pH 7.4; (C) 0.164 M phosphate buffer, pH 6.6; and (D) phosphate-citrate buffer, pH 6.6. The catecholase units of enzyme employed are indicated at each curve. The dotted lines show the volumes equivalent to 2 and 4 atoms of oxygen per molecule of dihydroxyphenylalanine.

caused no further increase in oxygen consumption. At pH 6.6, in 0.164 M phosphate buffer, increasing the enzyme concentration from 11 to 22 units per 2.8 ml. of reaction volume caused an increase in the oxygen consumed at 240 minutes from 125 to 165 μl.; further increase of enzyme concentration had no effect. In phosphate-citrate buffer at the same pH, increasing enzyme concentration to 44 units caused an increase of oxygen

consumed at 240 minutes to 155 μl.; further addition of enzyme had no effect.

The influence of buffer concentration upon oxygen consumption at approximately neutral pH is shown in Fig. 2. In these experiments the initial tyrosinase concentration was 88 units per 2.8 ml. to insure that oxygen consumption would be independent of enzyme concentration (Fig. 1). Fig. 2, A shows that, as buffer concentration was changed from 0.008 M (pH 7.6) to 0.164 M (pH 7.4) phosphate, the oxygen consumed at 240 minutes increased from 145 to 170 μl. As the buffer concentration was similarly varied at approximately pH 6.6 (Fig. 2, B), the oxygen consumed at 240 minutes increased from 112 to 165 μl.
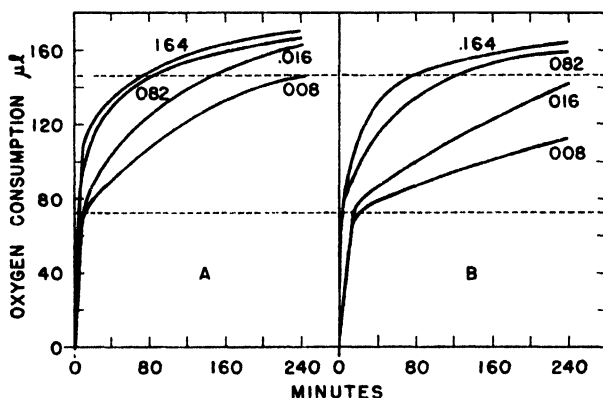
FIG. 2. The oxygen consumed during the oxidation of 0.65 mg. of dihydroxyphenyl-DL-alanine in the presence of 88 units of tyrosinase with increasing concentrations of phosphate (A) at pH 7.4 and (B) at pH 6.6. The dotted lines show the volumes equivalent to 2 and 4 atoms of oxygen per molecule of dihydroxyphenylalanine.

The effect of increasing enzyme concentration upon oxygen consumption and carbon dioxide evolution from 0.164 M phosphate buffer at pH 5.1 is shown in Fig. 3. The buffer capacity of phosphate at this pH is very low, but checks before and after the experiments described showed no variation. At concentrations above 11 units per 2.8 ml., approximately 74 μl. of oxygen (2 atoms per molecule of dihydroxyphenylalanine) are consumed rapidly (Curves A); a break in the consumption rate then occurs and the oxygen uptake continues at a much lower rate, but without reaching a definite limiting value within the period of observation, which extended to 18 hours. When the central well of the reaction vessels contained buffer instead of KOH, carbon dioxide accumulated in the gaseous phase. Curves B represent the course of volume changes in these vessels. In the presence of 44 or more units of enzyme a volume change equal to

consumption of approximately 74 μl. of oxygen took place rapidly. Little further change occurred in vessels containing 66 or more units of enzyme, indicating that the carbon dioxide produced equaled the oxygen con-
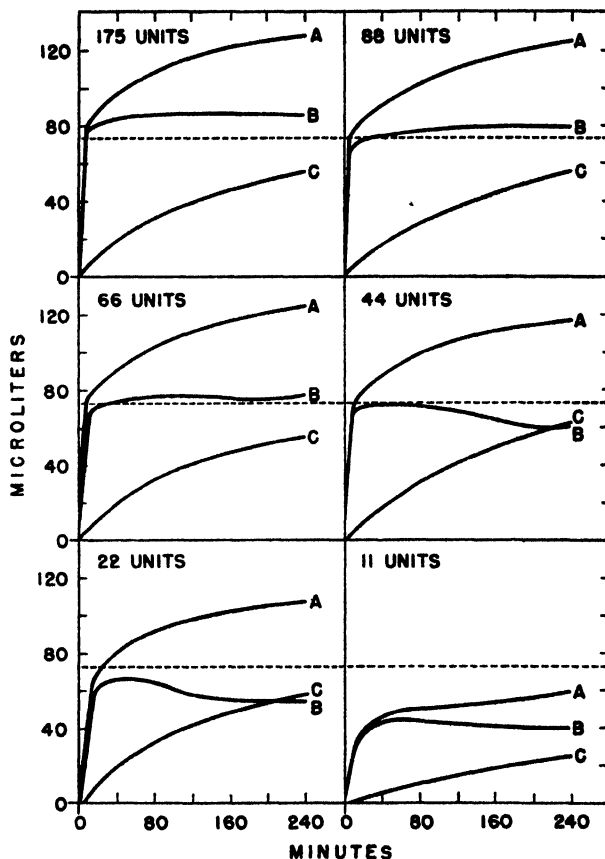
FIG. 3. The oxygen consumed and the carbon dioxide evolved during the oxidation of 0.65 mg. of dihydroxyphenyl-DL-alanine in 0.164 M phosphate buffer, pH 5.1, in the presence of increasing amounts of tyrosinase. The catecholase units of enzyme are indicated on each graph. Curves A represent oxygen consumption, Curves B the observed volume changes without absorption of carbon dioxide, and Curves C the carbon dioxide evolution calculated from the difference between Curves A and B. The dotted lines show the volume of oxygen or carbon dioxide equivalent to 1 molecule per molecule of dihydroxyphenylalanine.

sumed. The carbon dioxide evolution calculated from these sets of curves showed that, in the presence of 22 or more units, approximately 55 μl. were evolved in 240 minutes (Curves C).

The data in Figs. 1 and 3 referring to oxygen consumption at 240 minutes in 0.164 M phosphate buffers as a function of enzyme concentration have been recalculated as atoms of oxygen per molecule of dihydroxyphenylalanine in Fig. 4. This figure demonstrates that at 240 minutes 22 or more units of enzyme catalyze the uptake of 4.6 atoms of oxygen at pH 7.4 under the conditions of the experiment; 44 or more units catalyze the uptake of 4.5 atoms of oxygen at pH 6.6 and 44 or more units catalyze the uptake of 3.4 atoms of oxygen at pH 5.1.
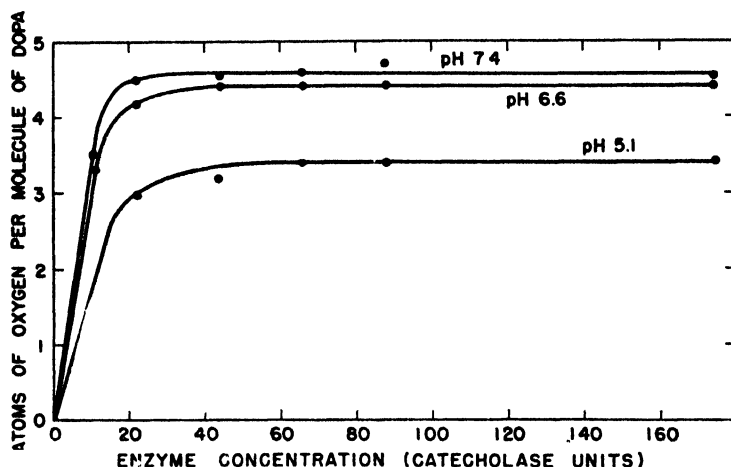
FIG. 4. The atoms of oxygen consumed per molecule of dihydroxyphenyl-DL-alanine with increasing tyrosinase concentration. The values were observed at 240 minutes of oxidation of 0.65 mg. of substrate dissolved in 2.8 ml. of 0.164 M phosphate buffers of the indicated pH.

The effect of buffer salts upon oxygen consumption and carbon dioxide evolution during the oxidation of 0.65 mg. of dihydroxyphenylalanine is shown in Fig. 5. The enzyme concentration was fixed at 88 units and the pH, 5.1, was held constant within the limits mentioned above. The buffer concentrations were varied as indicated on each curve. In 0.016, 0.082, and 0.164 M citrate buffers the oxygen consumption at 240 minutes was 122, 144, and 150 μl., respectively; the corresponding carbon dioxide evolutions were 43, 64, and 78 μl. Measurements of gaseous exchange in 0.016 M phosphate buffer at this pH were erratic, but at 0.082 and 0.164 M, 110 and 124 μl. of oxygen were consumed and 49 and 55 μl. of carbon dioxide were evolved in 240 minutes. In the corresponding concentrations of acetate buffer the values observed were 106, 110, and 132 μl. of oxygen consumed, and 43, 48, and 58 μl. of carbon dioxide evolved.

The effect of neutral salt is also depicted in Fig. 5. When reaction

mixtures either 0.016 or 0.164 M in citrate were made up to 0.164 M in sodium chloride (pH 5.2 and 5.0, respectively), the rate of oxygen consumption throughout the period of observation was depressed; at 240 minutes 93 and 110 μl. were taken up.

The effect of increasing buffer concentration upon the rate of carbon dioxide evolution from hallachrome[2] in the absence of enzyme was also observed. Solutions of hallachrome were prepared by oxidation of di-
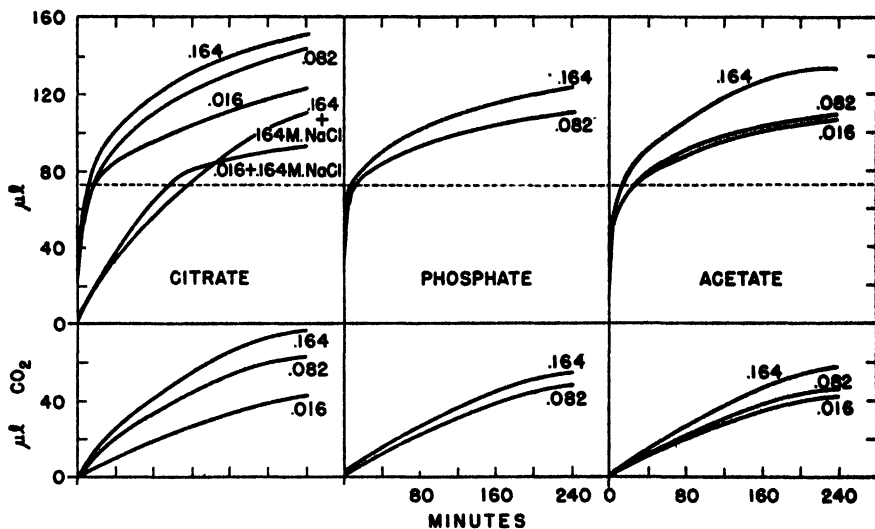
FIG. 5. The oxygen consumption and carbon dioxide evolution with increasing buffer concentration during the oxidation of 0.65 mg. of dihydroxyphenyl-DL-alanine at approximately pH 5.1 in the presence of 88 enzyme units. Buffer molarities are indicated on each graph. The effect of 0.164 M NaCl upon oxygen consumption in citrate buffers is also shown. The dotted lines indicate the volume equivalent to 2 atoms of oxygen per molecule of dihydroxyphenylalanine.

hydroxyphenylalanine with silver oxide (2, 3). Assuming quantitative conversion, concentrations were adjusted to 0.65 mg. of hallachrome per 2.8 ml. of 0.016 and 0.164 M citrate buffers adjusted to exactly pH 5.1. The carbon dioxide evolved was followed manometrically. Fig. 6 describes the results: carbon dioxide evolution from 0.164 M citrate solution was the more rapid.

The presence of 0.001 M concentrations of cupric, barium, or calcium ions did not affect the rate of carbon dioxide evolution during the enzymic

---

[2] When the term hallachrome is used in this paper, 2,3-dihydro-2-carboxyindole-5,6-quinone is indicated.

oxidation of dihydroxyphenylalanine in 0.016 M acetate buffer at pH 5.1. Mercuric ion at this concentration inhibited the consumption of oxygen. Zinc ion slightly catalyzed the evolution of carbon dioxide at a concentration of 0.005 M under these conditions.
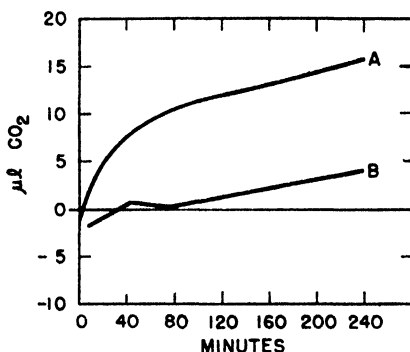
FIG. 6. The non-enzymic carbon dioxide evolution from solutions of 0.65 mg. of hallachrome in 2.8 ml. of citrate buffers, Curve A, 0.164 M and, Curve B, 0.016 M, both adjusted to pH 5.1.

No difference in the rates of oxygen consumption or carbon dioxide evolution was observed during the oxidation of dihydroxyphenyl-L-alanine and dihydroxyphenyl-DL-alanine in 0.164 M acetate solutions at pH 5.1 in the presence of 88 units of enzyme.

DISCUSSION

During the oxidation of a fixed weight of dihydroxyphenylalanine an increase in the concentration of tyrosinase, within limits, causes an increase in the amount of oxygen consumed within a given time (Figs. 1, 3, 4). Increase in enzyme concentration beyond these limits results in no further increase in oxygen consumption. The rate of consumption of the first 2 atoms of oxygen per molecule of dihydroxyphenylalanine is generally too high to permit accurate measurements of the effect of enzyme concentration upon it (Figs. 1, 3), but the rate and amount of oxygen consumed beyond this point may be satisfactorily determined. Since there is a range in which the amount and rate of oxygen consumption beyond the second atom is not affected by changes in enzyme concentration, it is probable that the velocity of a non-enzymic step is the rate-controlling factor. This step will presently be shown to be the decarboxylative rearrangement of hallachrome.

In the range of concentration in which the amount of enzyme limits the oxygen consumption, the behavior observed parallels that of catechol

(6) and is caused by inactivation of tyrosinase during the reaction. In Figs. 1, 3, and 4, it is shown that the amount of enzyme necessary to catalyze limiting oxygen consumption is influenced by the electrolytes in solution.

In the presence of amounts of enzyme optimal for all conditions (88 units) a variation of the ionic composition of the solution will also affect the rate and amount of oxygen consumed beyond the first 2 atoms (Figs. 2, 5). At low buffer concentrations either at pH 7.6 or 6.9 a break in oxygen consumption rate is observed at 2 atoms of oxygen per molecule of dihydroxyphenylalanine. This is the amount of oxygen required to convert dihydroxyphenylalanine into hallachrome, and the speed with which it is consumed, in the presence of excess enzyme, together with the marked change of rate at the end of this phase, indicates that hallachrome is formed quantitatively under the conditions utilized.

The change in rate at 2 atoms of oxygen is more clearly demonstrable at pH 5.1 and can then be correlated with the initiation of carbon dioxide evolution (Figs. 3, 5). Accordingly, carbon dioxide must evolve after hallachrome is formed, in agreement with the hypothesis (1, 2). Furthermore in the presence of 66 or more units of enzyme Curves B of Fig. 3 remain approximately level after the initial consumption of 2 atoms of oxygen, indicating that roughly 1 molecule of oxygen is being utilized for each molecule of carbon dioxide formed. This relationship shows that, in so far as the manometric method reveals, decarboxylation and rearrangement of hallachrome take place simultaneously.

The rates and amounts of oxygen consumed and carbon dioxide evolved are dependent not only upon enzyme concentration (up to optimal levels) but also upon the concentration and kind of buffer employed (Fig. 5). The slight increases of pH due to dilution of buffers discussed under "Experimental" should increase oxygen consumption according to the results shown in Fig. 4, but the influence of electrolyte dilution in the opposite direction is apparently greater, because dilution of buffer solutions under these conditions caused a decrease in rate of both oxygen consumption and carbon dioxide evolution.

The rate of carbon dioxide evolution observed in the presence of a series of citrate, phosphate, and acetate buffers has been plotted as a function of the concentration of unchanged hallachrome (Fig. 7). Linear relationships are found to hold, indicating that decarboxylation is a reaction of the first order with respect to hallachrome concentration. By plotting the specific first order rate constants so derived against anion concentration an approximately linear relationship is again found (Fig. 8). No regularity is observable if the specific rate constants are plotted against cation concentrations. The decarboxylative rearrangement of hallachrome is

accordingly subject to general base catalysis, but the analysis cannot be carried farther in terms of the Bronsted law because of the depressive action of neutral salt upon the rate of formation of hallachrome (Fig. 5) In the latter connection, Lea (9) has reported an inhibiting effect of NaCl on melanin formation. The present results indicate that the effect is probably due to the influence of chloride ion upon enzymic steps in the melanogenic sequence.
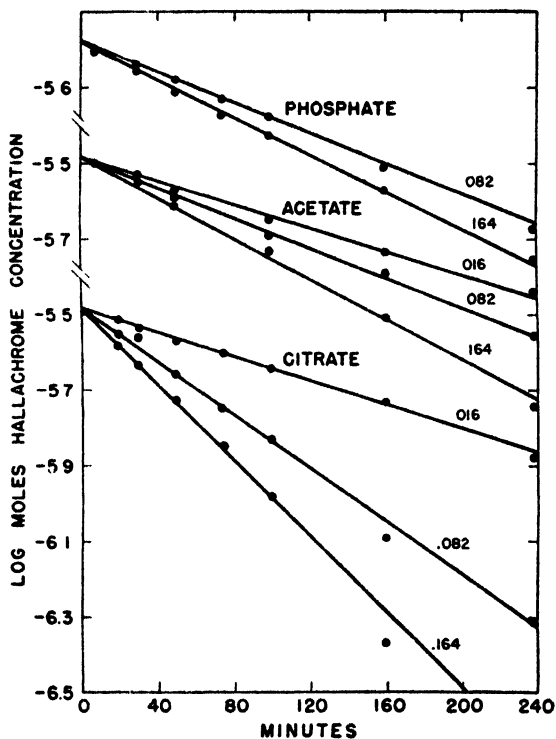
FIG. 7. The first order character of the decarboxylation of hallachrome, from the data of Fig. 5. Carbon dioxide evolution is expressed as moles of unchanged hallachrome. The molarity of the buffers is indicated on each curve.

The effect of anions upon the decarboxylative rearrangement of hallachrome does not involve the presence of a decarboxylase in mushroom tyrosinase, because the same effect occurs in the absence of enzyme (Fig. 6). In the absence of a catechol oxidase, however, hallachrome may be reduced by the products of its own rearrangement; this loss of hallachrome is reflected in a low rate of carbon dioxide evolution. In addition to the anion effect upon non-enzymic decarboxylation, increase of enzyme con-

centration beyond 22 units per reaction volume at pH 5.1 failed to in-
crease the rate or amount of carbon dioxide evolved (Fig. 3).

The oxygen consumed when dihydroxyphenylalanine is oxidized in the
presence of tyrosinase has been measured by Enselme and Vigneau (5)
and Raper and coworkers (3, 4). The highest value reported was 4.1
atoms per molecule, observed at pH 8 as an unchanging quantity. In
this study higher values were observed. The atoms of oxygen consumed
and the molecules of carbon dioxide evolved per molecule of dihydroxy-
phenylalanine in the presence of excess enzyme have been calculated from
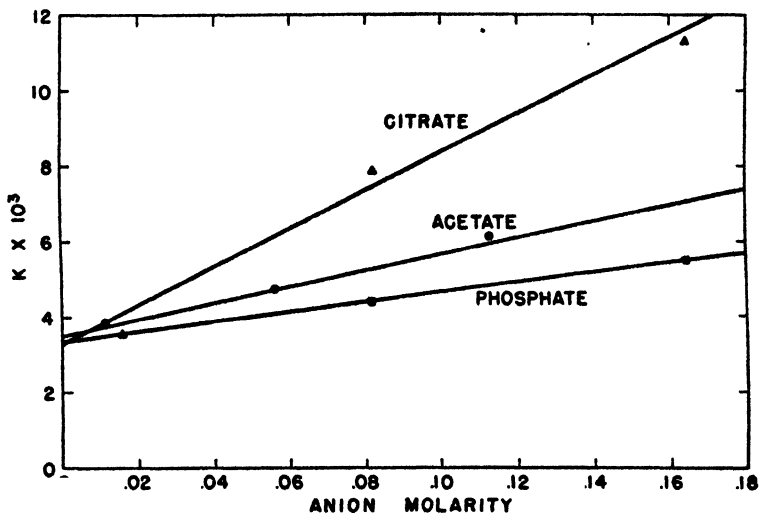
Fig. 8. The relationship between specific rate constants of hallachrome decar-
boxylation in citrate, phosphate, and acetate buffers and anion concentration,
calculated from the data of Fig. 7.

the data given above and are listed in Table I as functions of pH and buffer
concentration. At 240 minutes from 2.9 to 4.6 atoms of oxygen were
required; at pH 5.1 the carbon dioxide evolution amounted to 0.6 to 1.0
molecule. Gaseous exchange continued beyond this time and at 18
hours much higher values were observed, but these reflected respiration
of microorganisms to an undeterminable extent. In general, increasing
pH led to higher oxygen consumption. Increasing buffer concentration
led to both higher oxygen consumption and carbon dioxide evolution.

The amounts of oxygen required and carbon dioxide set free limit struc-
tures assignable to melanins formed by the enzymic oxidation of dihydroxy-
phenylalanine. In the present investigation the extent of gaseous ex-
change was dependent upon time and the electrolytic nature of the solution

even in the presence of excess enzyme. Utilization of more than 4 atoms of oxygen per molecule was readily realized (Fig. 4), but the point at which enzymic oxidation stopped and autoxidation commenced is not determinable from the data, nor is the point, if any, at which the polymeric product reaches its greatest complexity and commences to break down into simpler substances. Dopa melanin cannot therefore be regarded as a homogeneous reaction product, the composition of which is independent

TABLE I

Atoms of Oxygen Consumed and Molecules of Carbon Dioxide Evolved per Molecule of Dihydroxyphenylalanine

Varying pH and buffers but enzyme concentration fixed at 88 units per reaction volume of 2.8 ml.

Buffer	pH	O 240 min.	CO2 240 min.
0.164 M phosphate	5.1	3.4	0.7
0.082 " "	5.1	3.0	0.7
0.164 M acetate	5.1	3.6	0.8
0.082 " "	5.1	3.0	0.7
0.016 " "	5.1	2.9	0.6
0.164 " citrate	5.1	4.1	1.0
0.082 " "	5.3	3.9	0.9
0.016 " "	5.5	3.4	0.6
Citrate-phosphate	5.1	4.1	1.0
0.164 M phosphate	6.6	4.5	
0.082 " "	6.6	4.3	
0.016 " "	6.8	3.8	
0.008 " "	6.8	3.0	
Citrate-phosphate	6.6	4.3	
0.164 M phosphate	7.4	4.6	
0.082 " "	7.4	4.5	
0.016 " "	7.6	4.3	
0.008 " "	7.6	3.9	
Citrate-phosphate	7.4	4.6	

of conditions of preparation. These conditions should therefore be specified precisely in any study of the structure of melanin to permit reproducibility of the substance under investigation.

SUMMARY

1. Increase in tyrosinase concentration causes increase in over-all oxygen consumption during the enzymic oxidation of dihydroxyphenylalanine only within limits. Further increase leads to no further increase in oxygen consumption at a given time.

2. The electrolytic composition of the solution influences the amount of enzyme necessary to catalyze limiting consumptions of oxygen, as well as the total consumption of oxygen and evolution of carbon dioxide.

3. The decarboxylative rearrangement of hallachrome is base-catalyzed and controls the rate of oxygen consumption beyond the 2nd atom.

4. At 240 minutes under the conditions employed 2.9 to 4.6 atoms of oxygen were consumed and 0.6 to 1.0 molecule of carbon dioxide was evolved per molecule of dihydroxyphenylalanine.

BIBLIOGRAPHY

1. Raper, H. S., *J. Chem. Soc.*, 125 (1938).
2. Mason, H. S., *J. Biol. Chem.*, 172, 83 (1948).
3. Duliere, W. L., and Raper, II. S., *Biochem. J.*, 24, 239 (1930).
4. Heard, R. D. H., and Raper, H. S., *Biochem. J.*, 27, 36 (1933).
5. Enselme, J., and Vigneau, J. L., *Bull. Soc. chim. biol.*, 27, 337 (1945).
6. Wright, C. I., and Mason, H. S., *J. Biol. Chem.*, 185, 45 (1946).
7. Mallette, M. F., Lewis, S., Ames, S. R., Nelson, J. M., and Dawson, C. R., *Arch. Biochem.*, 16, 283 (1948).
8. Miller, W. H., Mallette, M. F., Roth, L. J., and Dawson, C. R., *J. Am. Chem. Soc.*, 66, 514 (1944).
9. Lea, A. J., *Nature*, 155, 428 (1945).

SUMMARY

1. A rapid micromethod for short chain carboxylic acid esters, lactones, and anhydrides is based on their ability to react with hydroxylamine quantitatively in an aqueous alkaline solution.

2. Acetylcholine may be determined by the method in the presence of large excess of acetate and choline.

3. Conditions which determine the scope of action of hydroxylamine as a trapping reagent have been investigated.

BIBLIOGRAPHY

1. Nachmansohn, D., and Weiss, M. S., *J. Biol. Chem.*, **172**, 677 (1948).
2. Feigl, F., Anger, V., and Frehden, O., *Mikrochemie*, **15**, 9 (1934).
3. Davidson, D., *J. Chem. Education*, **17**, 81 (1940).
4. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945).
5. Hill, U., *Ind. and Eng. Chem., Anal. Ed.*, **18**, 317 (1946).
6. Hill, U., *Anal. Chem.*, **19**, 932 (1947).
7. Bauer, F. C., Jr., and Hirsch, E. F., *Arch. Biochem.*, **20**, 242 (1949).
8. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **161**, 415 (1945).
9. Spec , J. F., *J. Biol. Chem.*, **168**, 403 (1947).
10. Elliot, W., *Nature*, **161**, 128 (1948).
11. Elliot, W., and Gale, E., *Nature*, **161**, 129 (1948).
12. Chantrenne, H., *Compt. rend. trav. Lab. Carlsberg*, **26**, 231 (1948).
13. Hantzsch, A., and Desch, C., *Ann. Chem.*, **323**, 1 (1902).
14. Bergmann, M., du Vigneaud, V., and Zervas, L., *Ber. chem. Ges.*, **62**, 1909 (1929).
15. Clarke, H. T., Johnson, J. R., and Robinson, R., The chemistry of penicillin, Princeton, 428, 1029 (1949).
16. Lipmann, F., *Advances in Enzymol.*, **6**, 257 (1946).

CHEMICAL ESTIMATION OF VITAMIN E IN TISSUE AND THE TOCOPHEROL CONTENT OF SOME NORMAL HUMAN TISSUES*

By MARY LOUISE QUAIFE AND MEI YU DJU

(From the Research Laboratories of Distillation Products, Inc., Rochester, New York)

A method for the chemical assay of vitamin E in animal tissues is described here which appears to be accurate and precise and to give values for biologically active tocopherol. It is based on the procedure previously described for vitamin E in foods (1) with certain modifications. In addition, the vitamin E content of a number of tissues from normal humans of both sexes has been determined and is presented.

The method of analysis for vitamin E in foods consists of the following steps: (1) vacuum dehydration from the frozen state; (2) homogenization; (3) extraction of vitamin E (and lipides); (4) concentration of vitamin E by molecular distillation; (5) removal of interference due to carotenoids by hydrogenation; (6) colorimetric assay for tocopherols. These steps were described in detail or referred to in a previous paper (1). For assay of animal tissues certain changes in the procedure are required. These changes and the reasons for them are given in the following section.

Discussion of Method

While the method which has been described for analysis of vitamin E in foods is considered satisfactory for materials of a low fat content, *e.g.* fruits, vegetables, cereals, it is not entirely satisfactory for those of a high fat, low tocopherol content, *e.g.* animal tissues. This refers chiefly to the methods of sampling, homogenization, and extraction.

Preparation of Sample—For vegetables and fruits with high water and low fat content, it is very convenient to dehydrate in the frozen state prior to homogenizing in a mechanical grinder (Wiley mill). This reduces the bulk of the sample, with consequent concentration of tocopherol content, and facilitates grinding of the sample. It also speeds up the extraction step, since dehydration is a necessary preliminary to lipide extraction.

However, for animal tissues these measures are not adequate. Since the water content is relatively low, little concentration of tocopherol is obtained. The dehydrated sample cannot be ground mechanically to a state of fine dispersion because of "smearing" due to high fat content.

* Communication No. 141.

263

The required coarse grinding makes it difficult to obtain samples which are representative of the whole or which are equal in vitamin E content. As the coarse ground material is less penetrable to extraction solvents than that which is finely ground, a much longer extraction period is required. These considerations are reflected in the higher coefficients of variation found for animal than for vegetable products when assayed by the food method (1).

Homogenization—For ease of sampling, a dry method of homogenizing is preferred. This rules out the method of grinding tissue under solvent (*e.g.*, in alcohol with the use of sand). The Waring blendor with solvent gives excellent homogenization, but it has been avoided for several reasons. (1) It does not allow simultaneous extraction of multiple samples. (2) It is not easy to use with organic solvents. Leakage frequently occurs through the gasket in the base and quantitative transfers are difficult to make. (3) "Foaming solvent" mixtures frequently give rise to difficulties with emulsion in the next stage: partition between water-alcohol and petroleum ether layers. (4) Relatively enormous quantities of solvents are required for small amounts of tissue. Since petroleum ether must be purified (2), owing to impurities which give an Emmerie and Engel (3) reaction, this involves extra work.

The advantage of using the Waring blendor is that it is a cold extraction technique. In cases in which heat appears to accelerate loss of vitamin E (low vitamin E, high fat, partially oxidized tissue; see below) it may be necessary to use such extraction despite its disadvantages.

The following "dry" homogenization method has been adopted. The tissue is cut in pieces of moderate size, which are placed in wire gauze and dipped in liquid nitrogen to freeze. The frozen tissue is transferred to an iron mortar which is surrounded by a solid carbon dioxide-acetone mixture. The pestle is chilled by placing it in the mortar or dipping in the liquid gas. A piece of ordinary wire gauze with a hole in the center surrounds the pestle and covers the mortar to keep pieces of tissue from popping out during grinding. The tissue is ground until it can be sieved through wire gauze (12 mesh per inch), previously chilled by dipping in liquid nitrogen, into a beaker which is kept in a dry ice-acetone bath. After all of the sample is ground and sieved, it is mixed and individual samples are weighed into Soxhlet thimbles. After this, no special pains need be taken to keep it frozen, since sampling has been accomplished. However, to minimize loss of vitamin E or oxidation of fat in the sample, it is kept at $-22°$ till assay or, preferably, assayed immediately. This homogenization procedure has been applied to nearly every variety of animal tissue with apparently successful results.

Extraction—Extraction of tissues for vitamin E is essentially an extrac-

tion of lipide.   For the reasons stated above the method of Waring blendor extraction with "foaming solvent" is not considered desirable for multiple assay.   The extraction with hot ethanol, by use of a multiple unit apparatus (1), has been retained.   Owing to the coarser subdivision of the sample, the extraction would be expected to take longer than that of the more finely divided food samples.

Beef liver was used as the experimental tissue.   It should contain as many if not more interfering materials than any other tissue.   Liver was
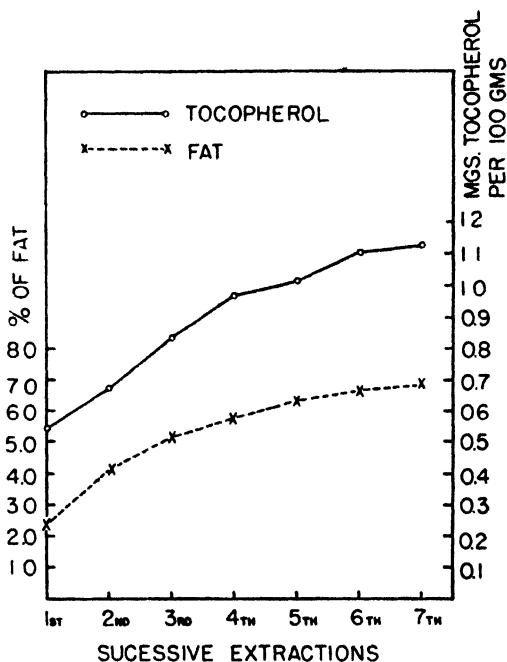
FIG. 1. Tocopherol and fat content of successive 2 hour extracts of beef liver. The values are cumulative.

obtained fresh from the slaughter-house and kept at $-22°$ till used, within 1 month.   All extraction experiments were performed in quadruplicate.

Samples were homogenized as described above and subjected to successive 2 hour extraction periods.   The extracts were treated as in the food method for vitamin E; i.e., reextracted into purified Skellysolve B, molecularly distilled, hydrogenated, and assayed colorimetrically (1).   The results (Fig. 1) show that an extraction period of several hours is required and that vitamin E extraction parallels that of fat.

After apparent exhaustive extraction, concentrated hydrochloric acid (1 ml.) was added to the extraction solvent (50 ml. of ethanol) and two

further extractions made; little or no increase in apparent tocopherol resulted. This provides further evidence (4, 5) that tocopherol does not occur in animal tissue in some bound form which can be set free by acidified solvent extraction.

The liver from a rat which had been fed tocopherol synthesized with radiocarbon (C^{14}) was homogenized and exhaustively extracted in the same manner as the beef liver. Counts of radioactivity as well as tocopherol assays were made on successive extracts[1] (Fig. 2). That these parallel
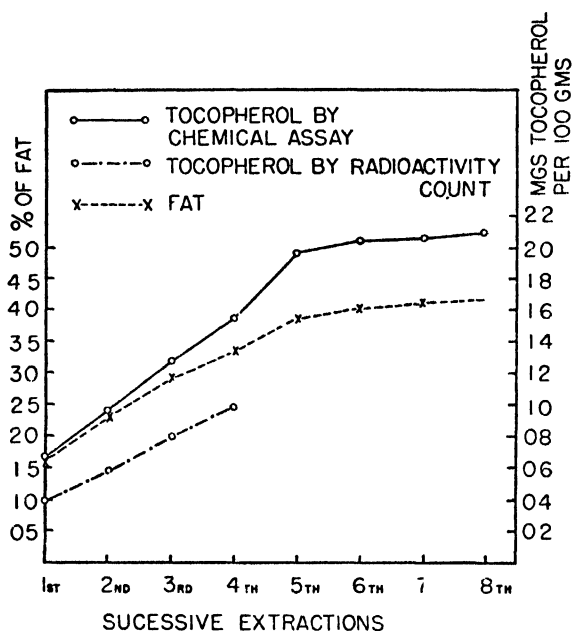
FIG. 2. Tocopherol and fat content of successive 2 hour extracts of liver of rat which had been fed radioactive α-tocopherol. The values are cumulative.

one another is strong evidence that the reducing material which is contained in the successive extracts is not extraneous material but true tocopherol. Addition of HCl to the residue of the rat liver, as in the beef liver experiment, also failed to extract significant amounts of tocopherol.

Experiments were next made to test the length of time required for complete extraction. Although in some experiments this occurred in 18 hours or less (Fig. 3), in several others it took longer. On the basis of

[1] The lipide extract was spread in a thin film and counted with a thin window Geiger tube. The amount of tocopherol was calculated from a calibration curve prepared with the same radioactive tocopherol which was fed. We are indebted to Mr. E. M. Shantz for this procedure.

these data a 24 hour period was provisionally adopted. However, for most of the assays reported an additional 2 hour extraction was made to check completeness of the first. In all cases a period of 24 hours was found to be sufficient.

Additional experiments were made to test completeness of extraction in the 24 hour period. In one case the beef liver residue after 24 hours of ethanol extraction was dried (2 hours at 80°), gound in a Wiley mill to pass a 40 mesh screen, and reextracted with ethanol for 2 hours. The results showed no residual tocopherol. In another experiment beef liver
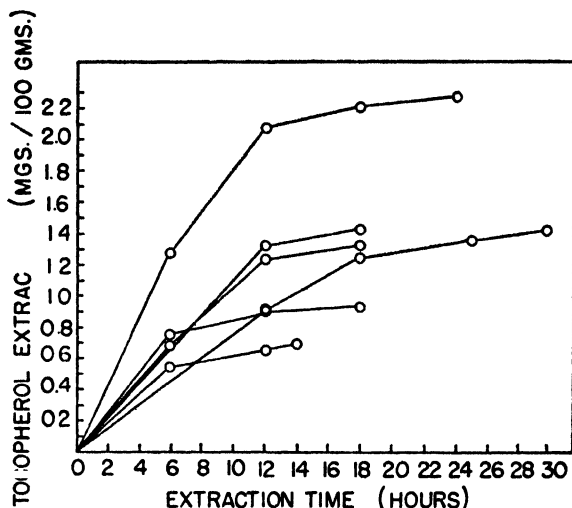
Fig. 3. Extraction of beef liver for successive periods. The ordinate represents cumulative tocopherol concentration in the liver. The abscissa gives cumulative extraction time.

was homogenized and extracted 24 hours with ethanol. The residue was then extracted 24 hours longer with boiling purified Skellysolve B. Again, the amount of tocopherol extracted was less than 2 per cent that of the total in the first extract.

The experiments described are considered to have established the adequacy and completeness of the extraction step for tocopherols in tissue. Since the remainder of the assay procedure consists of steps previously established, it remains to show satisfactory recovery and degree of precision of the complete analytical method for animal tissues. These are described below.

Recovery—Recovery experiments were made by assaying tissues with and without added tocopherol; tocopherol was added at a concentration about equal to that in the tissue. Ethanol solutions of pure, natural γ-tocopherol

were added to the extraction solvent and the entire analytical procedure carried out, including a 24 hour extraction period. Control colorimetric assays were made of equal amounts of the untreated tocopherol solution. The results show quantitative recovery (Table I). Recovery experiments were run routinely on many of the human tissues whose vitamin E values are reported here. Results of these are included in Table II, and they show quantitative recovery.

TABLE I

Recovery of Tocopherol by Vitamin E Assay Method for Tissue*

Sample	Extraction for 24 hrs			2nd extraction. 2 hrs.	
	Total tocopherols†	γ- + δ-tocopherols by diazo assay‡	Fat	Total tocopherols†	Fat
	mg. per 100 gm	*mg. per 100 gm*	*per cent*	*mg. per 100 gm.*	*per cent*
Beef liver (a)	1.40 ± 0.04 (s.d.)	0.000	5 08	0.00	0.09
" " + added γ-tocopherol (b)	2 17 ± 0 04 (s.d.)	0 855 ± 0 016 (s.d.)	5 01	0 00	0.10
Added γ-tocopherol (control) (c)	0.829	0.826			
Recovery§	93	103			

* The data, which are typical, were obtained from a single experiment. (a) and (b) values each represent the mean assay value for four 10 gm. aliquots of homogenized fresh beef liver.

† Emmerie and Engel (3) color reaction, in which ferric chloride and α,α-dipyridyl are used.

‡ The method which involves coupling of γ- and δ-tocopherols with diazotized o-dianisidine to give colored compounds.

§ Per cent recovery $= \dfrac{(b) - (a)}{(c)} \times 100$.

Poor recoveries were obtained in some instances when old samples of tissue were used (values not included in Table II). It is again emphasized that to obtain good recovery it is necessary that the tissue be fresh and unoxidized. Even tissues from freshly killed animals which had been maintained in a vitamin E-deficient state for a prolonged period gave low recovery values. This may mean that the tissues had become rancid *in vivo*. Possibly the chemical assay in such cases is a fair measure of the amount of vitamin E biologically available, since it is known that peroxidized fats increase the requirement for vitamin E. However, for an accurate chemical assay of the amount of tocopherol in such tissues a cold extraction step may be preferable if loss during assay is to be avoided.

Precision—Precision of the analytical method is good as shown by data on assays made on replicate portions of the homogenized tissue samples. For example, the data in Table I show coefficients of variation of the mean for quadruplicate assay of beef liver equal to 2.9 per cent. Simultaneous

TABLE II

Vitamin E in Normal Human Tissues

Subject	Tissue	Fat	Tocopherol content		Recovery of added γ-tocopherol, Emmerie and Engel method
			Total	γ- + δ-tocopherol	
		per cent	mg. per 100 gm. tissue	mg. per gm. fat	mg. per 100 gm. tissue	per cent
Male, 30 yrs. old	Muscle, pectoralis major	6.31	1.32	0.209	<0.27	94.9
	" abdominal wall	3.90	0.62	0.159	<0.05	
	Liver	6.12	2.49	0.407	<0.05	94.3
	Fat, abdominal, subcutaneous	83.2	24.7	0.297	2.63*	91.1
	†	81.1	29.2	0.359	3.33*	
	Heart	3.36	1.11	0.331	<0.09	98.6
	Testis	2.34	2.83	1.21	<0.08	93.7
	Kidney	6.59	0.80	0.122	<0.19	97.6
	Pancreas	10.29	5.49	0.533	<0.13	99.6
	Lung	3.79	1.16	0.305	0.34‡	
	Spleen	2.49	1.88	0.757	0.56‡	
Female, 43 yrs. old	Muscle, rectus abdominis	6.06	1.56	0.257	<0.11	96.0
	" psoas	4.33	3.80	0.877	1.02*	
	Liver	5.53	2.19	0.396	<0.14	96.2
	Fat, abdominal, subcutaneous	81.8	49.5	0.605	18.6*	
	" perirenal	62.6	39.2	0.626	11.5*	
	Heart	3.31	1.28	0.387	<0.14	106.6
	Uterus	1.27	1.47	1.16	0.31*	98.0
	Kidney	4.56	3.32	0.727	0.85*	
	Pancreas	17.4	10.6	0.609	3.75*	
	Spleen	9.21	4.70	0.510	1.92*	

* The values represent true γ- or δ-tocopherol as shown by the authentic spectra of the coupling products with diazotized *o*-dianisidine which were obtained in the tissue assays.

† On bioassay found to contain 34 mg. of *d,α*-tocopherol per 100 gm. of tissue.

‡ Probably not true γ- or δ-tocopherol. The diazotized dianisidine-coupling products had atypical spectra.

assays of six replicate samples of fresh beef liver were made on two occasions for comparison with bioassay. (Samples containing added tocopherol were also included and showed quantitative recoveries.) For the two sets of six samples the coefficients of variation of the mean were 2.0 and 1.1 per cent, respectively. These coefficients of variation of the mean

refer to total tocopherols in the tissue. At comparable levels of concentration, γ- plus δ-tocopherol assays show as good precision. These tocopherols are usually present in relatively small amounts in tissue samples, however, which reduce somewhat the precision of their measurement.

Accuracy—Bioassay comparisons with chemical assays were made previously for several foods by use of, essentially, the chemical method here described for tissues. In addition, comparative assays for total tocopherols were made by two independent chemical methods and in a few cases by direct comparison with the extinction value ($E_{290-300 \, m\mu}$) of the food extract due to tocopherol itself; these showed good agreement (1). The reliability of the tissue method probably exceeds that of the food method, since the tissue antioxidants which are lipide-soluble have been shown to be chiefly, if not exclusively, the tocopherols (6).

In addition, bioassays of certain animal tissues have been made to compare biopotency with the chemically determined α-tocopherol content, *i.e.* total tocopherols minus (γ- + δ-) tocopherols. The results have shown good general agreement between the two and have helped establish the chemical method as a reliable measure of vitamin E content.

Results

Vitamin E Content of Human Tissues—Certain tissues, freshly obtained from accident cases of apparently normal persons,[2] have been assayed for vitamin E. The data are given in Table II.

Tocopherol levels based on fat content are quite similar in a variety of tissues. The highest values occurred in testis and uterus. These may be significant in view of the primary rôle of vitamin E in maintenance of testicular function in the male rat or in prevention of uterine dysfunction in the female rat.

In neither case did the vitamin E content of the heart exceed that of other tissues, either on a fat or fresh weight basis. This is contrary to the finding of Mason for tissues of rats which were fed on a vitamin E-rich diet (7).

Body fat appears to be the major storage site for vitamin E in the normal human. It accounts for more than 75 per cent of the tocopherol estimated to be contained in a human female subject and more than 50 per cent for the male subject (Table III).

Total amounts of tocopherols for a hypothetical 50 kilo woman and a 70 kilo man were estimated by using the tocopherol levels given in Table II. Organ weights and proportionate tissue weights were found in standard

anatomy text-books. The data are shown in Table III. The calculated values for total tocopherols in these cases are 8 and 3.4 gm., respectively.

α-Tocopherol is the predominant form of tocopherol found in the tissues. In the male subject the presence of γ- or δ-tocopherol could be established only in the subcutaneous fat. However, γ- or δ-tocopherols were present in appreciable amounts in most of the tissues of the female subject, to the extent of 20 to 40 per cent of the total tocopherols present. Their identity

TABLE III

Estimated Content of Total Tocopherols in Human Subjects

Tissue	Woman	Man
	mg.	*mg.*
Fat..	6180	1885
Muscle..	269	285
Blood..	45	64
Liver..	33	45
Pancreas...	10	7
Spleen......	7	4
Heart..	4	3
Kidney..	10	2
Uterus..	2	
Lung..		12
Testis......		2
Total..	6560	2309
Total on basis of 50 kilos .	8120	
" " " " 70 "		3440

was established by comparison of the spectra of the diazotized dianisidine-coupling products of the tissue extracts with those of pure γ- and δ-tocopherol. They were identical.

SUMMARY

A method for chemical assay for vitamin E in animal tissues is described. It shows good precision and recovery values, provided fresh tissues are used, and appears to give an accurate measure of both total and α-tocopherol content.

Values for vitamin E in certain tissues of two normal human subjects are given. Tissues with the highest concentration on a fat basis were testis and uterus. Fat was the major storage site of tocopherols in these human subjects. Tissues of the same individual possessed quite similar values on a fat basis. Although α-tocopherol was the predominant form

of tocopherol in the tissues, significant amounts of γ- and δ-tocopherols were found in many of them.

BIBLIOGRAPHY

1. Quaife, M. L., and Harris, P. L., *Anal. Chem.*, **20**, 1221 (1948).
2. Werner, E. A., *Analyst*, **58**, 335 (1933); quoted by Weissberger, A., and Proskauer, E., Organic solvents, Oxford, 137 (1935).
3. Emmerie, A., and Engel, C., *Rec. trav. chim. Pays-Bas*, **57**, 1351 (1938).
4. Hines, L. R., and Mattill, H. A., *J. Biol. Chem.*, **149**, 549 (1943).
5. Kaunitz, H., and Beaver, J. J., *J. Biol. Chem.*, **166**, 205 (1946).
6. Hanson, H. T., Barnes, R. H., Lundberg, W. O., and Burr, G. O., *J. Biol. Chem., Chem* , **156**, 673 (1944).
7. Mason, K. E., *J. Nutr.*, **32**, 71 (1942).

TISSUE-BOUND, ACID-LABILE PHOSPHORUS IN TOBACCO LEAVES, OAT COLEOPTILES, AND RAT LIVER*

By SAM G. WILDMAN, JEAN M. CAMPBELL, and JAMES BONNER

(*From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena*)

In much of the work on phosphorus metabolism in plants and animals, primary attention has been focused on the P which is easily extracted with cold acid. However, such an extraction procedure usually removes only about one-half of the total P contained in many tissues (1, 3, 8, 11). As a result of recent work with radioactive P^{32}, it has become increasingly apparent that the acid-insoluble P is subject to rapid turnover and hence is of interest in P metabolism. For example, Gest and Kamen (4) have shown with *Chlorella* that there is rapid turnover of the acid-insoluble P and that the rate of turnover is markedly accelerated during photosynthesis. Our own work, which will be presented elsewhere, indicates that auxin influences the rate of turnover of the acid-insoluble P during the growth of oat coleoptiles. It becomes of interest, therefore, to determine the nature of the substances in which such acid-insoluble P is found.

Various attempts have been made to characterize the bound P in tissues (5, 8), and as a result the general current view is that a large proportion of such P is bound to proteins, although there is little direct experimental evidence to suggest what proteins are the responsible agents. In a study of P metabolism of green spinach leaves (11), it was found that almost one-half of the total P contained in leaves is acid-insoluble. Further investigation revealed that a large part of the bound P is associated with a bulk protein of spinach cytoplasm. This protein, Fraction I, constitutes about 75 per cent of the total cytoplasmic proteins, is electrophoretically homogeneous, contains 0.3 per cent total P, and has the enzymatic properties of a phosphatase (10).

The P present in Fraction I protein can be separated from the protein into two distinct subfractions (11). When the purified protein is precipitated with ice-cold N trichloroacetic acid (TCA), and irreversibly denatured as a consequence, about 50 per cent of the total protein P is freed into the clear Supernatant I which remains after removal of the protein by centrifuging. The cold acid extraction comes to an end-point, since a second identical treatment removes less than 8 per cent as much P as the first. Extending the extraction period for as long as 8 hours does not sig-

nificantly increase the yield of P. Thus, there is a large amount of P associated with Fraction I protein which is cold acid-insoluble. If now the cold acid-extracted and precipitated protein is (thoroughly) suspended in more cold acid, heated for 2 minutes in a boiling water bath, immediately cooled to 0°, and the protein removed by centrifuging, then nearly all of the remaining bound P (80 per cent) appears in the clear Supernatant II. Less than 10 per cent of the total protein P remains in the protein precipitate after the successive cold and 2 minute hot acid extractions.

It is of interest to review briefly (11) some of the properties of the cold acid extract, Supernatant I, and the 2 minute hot acid extract, Supernatant II, prepared from purified Fraction I protein according to the scheme just described.

Supernatant I—All of the P in this subfraction appeared to be inorganic. Evidence for the presence of adenine was obtained spectrophotometrically and confirmed by quantitative bioassay with an *adenineless* mutant of *Neurospora* (7). Pentose was determined by the orcinol-HCl reagent (9). The molar ratio of adenine, pentose, and total P was approximately 1:2:2. Unexpectedly, 50 per cent of the inorganic P in this fraction was soluble in hot absolute alcohol or boiling ether, which suggests that it is not sufficient to describe the P soluble in organic solvents as phospholipide unless there is other supporting evidence for the identity of these substances.

Supernatant II—The subfraction which remains with the protein after cold acid extraction and is released after heating the protein for 2 minutes in acid contains adenine, pentose, and total P in the approximate molar ratio of 1:1:2. Of the total P contained in Supernatant II, less than 5 per cent is present as inorganic P. However, continued heating of the 1 N acid extract results in the liberation of one-half of the total P as inorganic P. The remainder only appears as inorganic P after total digestion of the materials contained in Supernatant II with concentrated H_2SO_4. Thus, the 2 minute hot acid extract contains organic P in the ratio of 1 mole of labile P to 1 mole of stable P.

The hydrolysis characteristics of the labile P in Supernatant II are similar to those found with high energy phosphorus-containing compounds such as adenosine triphosphate, etc.; 45 per cent of the labile P in Supernatant II is released as inorganic P by a heating period of 7 minutes in 1 N HCl, 82 per cent is released in 16 minutes, and all of the labile P is released in 32 minutes, since no further increase in inorganic P occurred after heating 64 minutes. There are then substantial amounts of P bound to Fraction I protein of spinach cytoplasm, and more significantly, one-half of the total bound P is of an acid-labile nature and on this account may be of the high energy type (6). It is also noteworthy that the P appears to be in combination with adenine and pentose, although the solubility of the phos-

phorus in the presence of barium salts suggests that the compound is not identical with adenosine diphosphate.

Whole spinach leaves were also examined by the P distribution method described above. Labile P compounds contained in leaves were immobilized against chemical change by sudden freezing of the leaves with liquid air; the leaf tissue was dried *in vacuo* from the frozen state. Distribution analysis made on the leaf powder indicated that 15 per cent of the total leaf P is acid-labile P bound to tissue constituents and not soluble in cold acid, but is released into a clear extract by heating the tissue in 1 N acid for 2 minutes. The 2 minute hot acid extract prepared from whole leaves contained adenine in the ratio of 1 mole of adenine to 2 moles of total P. Of the total dry weight of the leaf tissue, 0.6 per cent is found as adenine in the hot acid extract.

The above results with spinach leaves suggested the desirability of examining other plant tissues and an animal tissue for P distribution, and particularly for bound, acid-labile P.

Methods and Materials

Preparation of Tissue—Freshly cut plant tissue was frozen in liquid air and dried by the lyophil method. When dry, the tissue was ground to pass a 40 mesh screen and stored *in vacuo* over P_2O_5 in darkness until used for analysis. Rat liver powder was obtained through the courtesy of Dr. H. Borsook of this Institute. The freshly excised liver was immediately frozen in liquid air, ground to a fine powder, and dried by the lyophil method.

P Distribution Analysis—The dry tissue powder is weighed and transferred to a sintered glass funnel. 5 ml. of ice-cold, 1 N TCA are added to the sample, the mixture is stirred for several minutes, and the liquid is drawn through with suction. The process is repeated with another 5 ml. of cold TCA. The combined filtrates (Supernatant I) are evaporated to a small volume at 98°, and then subjected to total digestion with concentrated H_2SO_4 before being analyzed for inorganic P according to the method of Berenblum and Chain (2).

The cold acid-extracted tissue is quantitatively transferred to a testtube by washing with 1 N HCl and made to 5 ml. volume. The tube is then placed in a boiling bath for exactly 2 minutes with vigorous stirring, plunged into an ice bath, and shaken to insure rapid cooling. The tissue is separated from the extract by filtering on sintered glass. The tissue is washed with an additional 5 ml. of cold HCl. The combined filtrates (Supernatant II) are evaporated to a small volume, totally digested, and analyzed for P. The residue is retrieved from the sintered glass and also analyzed for total P.

Acid-Labile P in Supernatant II—This material is prepared by the 2 minute hot acid procedure just described. After filtering, aliquots are removed from the ice-cold filtrate and heated for various periods of time in a boiling bath, cooled immediately, and directly analyzed for inorganic P.

EXPERIMENTAL

Tobacco Leaves—The data in Table I indicate that only 58.5 per cent of the total P in tobacco leaves is extracted with cold acid. A second extraction (Supernatant IA) yielded only 2.4 per cent of the total P, indicating that the cold extraction procedure comes to an end-point. When the cold acid-extracted tissue is heated for 2 minutes in 1 N HCl, 21.3 per cent of the total tissue P is released into the clear Supernatant II. Thus,

TABLE I

Distribution of Phosphorus in Various Tissues

Tissue fraction	Treatment	Total per mg. tissue			Total tissue P		
		Tobacco	Oats	Rat	Tobacco	Oats	Rat
		γ	γ	γ	*per cent*	*per cent*	*per cent*
Whole leaves	Total digestion	6.73	6.20	11.25	100	100	100
Supernatant I	Cold TCA extract	3.94	3.84	5.22	58.5	62.0	46.4
" IA	2nd cold TCA extract	0.16	0.16	0.34	2.4	2.6	3.0
" II...	2 minute hot HCl extract	1.43	1.53	3.73	21.3	24.7	33.2
Residue	Tissue remaining after cold and hot acid extraction	1.20	0.46	1.43	18.0	7.4	12.7

the 2 minute hot acid treatment is sufficient to release over 50 per cent of the bound P. The residue after cold and hot extraction contained only 18 per cent of the original total P of the tissue.

The acid-labile character of the P contained in Supernatant II obtained from tobacco leaves is shown in Table II. Without further heating other than the 2 minutes required to release the bound P from the tissue, only 2.8 per cent of the total P released was detected as inorganic P. However, further heating for 5, 12, and 30 minutes released respectively 10, 34.3, and 50 per cent of the total P as inorganic P. The ratio of 32 minute-labile P to stable P is 1:1. Thus, as in spinach leaves, a substantial portion of the total P in tobacco leaves (10.7 per cent) is bound in the tissue as acid-labile P, and is not extracted by the cold acid extraction procedures customarily used in P metabolism studies.

Avena Coleoptiles—Etiolated *Avena* coleoptiles represent a plant tissue which is grown under quite different environmental conditions than are the

mature green leaves used in previous experiments. The data in Table I show, however, that the distribution of P in the *Avena* coleoptile is quite similar to that found for green leaves. Only 60 to 70 per cent of the total P is extracted with cold acid. A combination of cold acid extraction followed by a 2 minute hot acid treatment removed all but 7 to 8 per cent of the total P originally contained in the tissue.

Table II shows the acid-labile nature of the P contained in the 2 minute hot acid extract of *Avena* coleoptile tissue. Less than 2 per cent of the total P in this preparation was detected as inorganic P following the 2 minute hot acid treatment required to release the phosphorus from the tissue. However, when the 1 N acid extract was additionally heated for 5, 12, and 30 minutes, 6, 26, and 49 per cent of the total P was released as inorganic P. The ratio of stable P to 32 minute-labile P is 1:1. Thus, 9.2 per cent

TABLE II

Acid-Labile Character of Tissue-Bound P Contained in 2 Minute, Hot Acid Extract of Various Tissues

Time of additional heating in 1 N HCl	Inorganic P per mg. dry tissue			Labile P in tissue-bound P		
	Tobacco leaves	Oat coleoptiles	Rat liver	Tobacco leaves	Oat coleoptiles	Rat liver
min.	γ	γ	γ	*per cent*	*per cent*	*per cent*
0	0.04	0.02	0.21	2.8	1.7	5.6
5	0.14	0.07	0.70	9.8	6.0	18.8
12	0.49	0.31		33.3	26.5	
30	0.72	0.57	1.30	50.2	48.7	34.9

of the total P in *Avena* coleoptiles can be considered as acid-labile P bound to tissue constituents.

Rat Liver—Lyophilized rat liver powder was treated according to the distribution scheme described above. The results are also given in Table I. Only 49.4 per cent of the total tissue P was extracted by two successive treatments with cold TCA. The second cold acid extraction produced only 6.5 per cent as much P as the first extraction, indicating that the cold acid extraction comes to an end-point. When the cold acid-extracted tissue was heated for 2 minutes in 1 N HCl, 33.2 per cent of the total tissue P was released, an amount representing 72 per cent of the tissue-bound P. The tissue remaining after these two extractions contained less than 13 per cent of the original total P.

The data in Table II show the acid-labile nature of the bound P of rat liver tissue. Without further heating other than the 2 minutes in hot HCl required to release the bound P from the tissue, 5.6 per cent of the total bound P was found to be present as inorganic P. However, 5 minutes

further heating caused 18.8 per cent of the total bound P to be released as inorganic P, while a 30 minute heating period released 34.9 per cent of the total bound P as inorganic P. It is therefore clear that rat liver, like plant tissues, contains large amounts of tissue-bound acid-labile P which is not extracted by the classical methods used in P metabolism studies. Approximately 10 per cent of the total P in rat liver can probably be considered as potential high energy tissue-bound P.

The present results on rat liver closely parallel those obtained by Umbreit et al. (9) in so far as comparisons can be made. Assuming that liver contains 80 per cent water, we calculate that they extracted 5.57 γ of P per mg. of dry liver with cold TCA, compared to the result in Table I of 5.22 γ of P per mg. obtained in Supernatant I. This latter figure, however, represents only 46 per cent of the total P contained in liver tissue. It must therefore be concluded that the scheme proposed by the Wisconsin group deals with less than one-half the total P in liver, and neglects the remainder, 72 per cent of which can be released into solution by a 2 minute hot acid treatment, and 35 per cent of which appears to be acid-labile P. It would obviously be of interest to examine this material for adenine and pentose.

<center>DISCUSSION</center>

Investigation of the P bound to protein of Fraction I of spinach cytoplasm (10, 11) has led to the hypothesis that the P may be present as pyrophosphate linked to organic material which in turn is loosely combined with the protein as illustrated by the accompanying diagram. "R" pre-

<center>Fraction I Protein</center>

$$\text{PROTEIN} \underset{a}{-} ^{''}\text{R}^{''} \underset{b}{-} \text{O} - \text{P} \underset{c}{-} \text{O} - \text{P}$$

<center>2 min. hot acid extract</center>
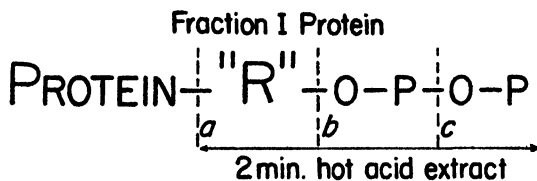
sumably represents adenine and pentose, compounds which are released along with pyrophosphate from the protein by the 2 minute hot acid treatment, and appear in Supernatant II. The mechanism by which bound organic P is released from the protein is thought to involve first the a linkage between the protein and "R." This linkage is completely disrupted by heating the protein for 2 minutes in 1 N acid, but the treatment is sufficiently mild, so that less than 5 per cent of the pyrophosphate linkage (b) is broken with the release of inorganic P. If, however, the materials in Supernatant II are heated for longer periods of time in 1 N acid, the b

linkage is completely broken.   The *c* linkage is resistant to this latter treatment and the remaining organic P is converted to inorganic P only by total digestion.

It must be emphasized that only 16 per cent of the weight of the 2 minute hot acid extract can be accounted for as adenine, pentose, and P even though the molar ratios of these three compounds are 1:1:2 respectively. Therefore, the substances in Supernatant II require further characterization before the suggested mechanism can be considered more than speculative.

The complete parallelism between the results obtained with purified Fraction I protein and the results obtained with whole spinach leaves leads us to believe that similar mechanisms are operative in releasing bound P

TABLE III

Efficiency of Release of Bound P in Various Tissues by 2 Minute, Hot Acid Treatment

Tissue	Tissue-bound P	Tissue-bound P released by hot acid in 2 min.	Labile P in tissue-bound P
	per cent	*per cent*	*per cent*
Spinach leaves....................	40	80	50
Tobacco "	42	57	50
Avena coleoptiles.................	38	77	49
Rat liver.........................	52	72	35

in the two instances.   That similar relations may also apply for other tissues is borne out by the data in Table III which is a summary of the work on spinach together with the essential information of this paper.   The data indicate the following: (1) In all tissues thus far examined, only 48 to 72 per cent of the total tissue P is extracted with cold acid.   (2) 57 to 80 per cent of the cold acid-insoluble or bound P of the tissue can be released by heating the tissue for 2 minutes in 1 N acid.   (3) From 35 to 50 per cent of the bound P is acid-labile P which can be released as inorganic P when extract (2) above is heated in 1 N acid.   (4) The remainder of the P in this same extract is resistant to treatment with hot 1 N acid and is not released as inorganic P until the material is completely digested.   The similarities in the distribution and properties of the bound P contained in such diverse tissues suggest that the present method may be a useful starting point in exploring the P relationships in other biological materials.

SUMMARY

1. A study was made of the distribution of phosphorus in spinach leaves, tobacco leaves, oat coleoptiles, and rat liver.   Cold acid extracted only 60, 58, 62, and 48 per cent respectively of the total tissue P.

2. If the P-containing tissue remaining after cold acid extraction is heated in 1 N acid for 2 minutes at 100°, then 80, 57, 77, and 72 per cent of the tissue-bound P is released from spinach leaves, tobacco leaves, oat coleoptiles, and rat liver respectively. The P so released is, however, not in the form of inorganic phosphate, but is still bound in organic combination.

3. Of the bound P released by the 2 minute hot acid treatment, 50, 50, 49, and 35 per cent can be liberated as inorganic P by an additional short heating period in 1 N acid.

4. Therefore, 15, 10.7, 9.2, and 10 per cent of the total P contained in spinach leaves, tobacco leaves, oat coleoptiles, and rat liver is acid-labile P which is bound to tissue constituents.

BIBLIOGRAPHY

1. Albaum, H. G., and Umbreit, W. W., *Am. J. Bot.*, **30**, 553 (1943).
2. Berenblum, I., and Chain, E., *Biochem. J.*, **32**, 295 (1938).
3. Emerson, R. L., Stauffer, J. F., and Umbreit, W. W., *Am. J. Bot.*, **31**, 107 (1944).
4. Gest, H., and Kamen, M. D., *J. Biol. Chem.*, **176**, 299 (1948).
5. Juni, E., Kamen, M. D., Reiner, J. M., and Spiegelman, S., *Arch. Biochem*, **18**, 387 (1948).
6. Lipmann, F., *Advances in Enzymol.*, **1**, 99 (1941).
7. Mitchell, H. K., and Houlahan, M. B., *Federation Proc.*, **5**, 370 (1946).
8. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945).
9. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1945).
10. Wildman, S. G., and Bonner, J., *Arch. Biochem.*, **14**, 381 (1947).
11. Wildman, S. G., Campbell, J. M., and Bonner, J., *Arch. Biochem.*, in press.

THE INHIBITION OF ALKALINE PHOSPHATASE
BY BERYLLIUM*

By FRIEDRICH W. KLEMPERER, JOSEPH M. MILLER, and CAROLINE
J. HILL

(From the Department of Biochemistry of the Trudeau Foundation for the Clinical
and Experimental Study of Pulmonary Disease, Trudeau, New York)

Recent clinical reports of pulmonary disease occurring in workers in the beryllium industry have stimulated new interest in the biochemical action of this element (for a complete bibliography, see (1)). Although it has not been possible to produce in animals pathological conditions identical with those seen in beryllium workers, experimental administration of beryllium and its compounds has resulted in a variety of lesions involving various organs and tissues. Of these lesions those affecting the skeletal system are of prime biochemical interest. Experimental rickets have been produced by feeding a diet which contains an excess of beryllium over the amount of phosphate ingested (2-5). Most authors are agreed that in this case the production of rickets is due, at least in part, to the low solubility of beryllium phosphate which, precipitated in the intestine, becomes unavailable for absorption. Nevertheless, *in vitro* experiments by Sobel *et al.* (5) indicate that a local factor is also operative in preventing calcification in beryllium rickets. A different effect of beryllium on bones has been demonstrated in rabbits by Gardner and Heslington (6) who observed eburnation with subsequent tumor formation following injection of sparsely soluble beryllium compounds. In view of the rôle which phosphatase plays in the formation of bone, it appeared possible that disturbances of normal ossification might depend on interference of beryllium with the action of this enzyme. Guyatt, Kay, and Branion (2), without giving experimental evidence, stated that "Be^{++} was occasionally strongly activating" to phosphatase. Cloetens (7), who studied the effect of various metals on alkaline phosphatase, reported inactivation of the enzyme by 10^{-3} M solution of beryllium chloride. Greenstein *et al.* (8) observed an inhibiting effect of beryllium on the formation of ammonia and inorganic phosphate from nucleic acids by crude tissue extracts. The following report represents an investigation of the action of beryllium on phosphatase.

All enzyme solutions used in the following experiments were prepared from defatted and dehydrated hog kidney powder, obtained from the Viobin Corporation. The powder was extracted for 5 minutes with 100 parts of water, centrifuged, and the clear extract dialyzed against distilled water for a period sufficient to remove all traces of inorganic phosphate. An inactive precipitate which formed during dialysis was separated by centrifugation. The beryllium solutions were made from either commercial beryllium sulfate dissolved in water or from beryllium chloride prepared by dissolving the purest obtainable metal[1] in hydrochloric acid. No difference was detected between the action of these two salts. Estimation of the activity of alkaline phosphatase was essentially as described by Bodansky (9).

For each determination, 5 ml. of a solution containing 50 mg. of sodium β-glycerophosphate in 0.04 M veronal solution were added to the indicated amount of beryllium and the volume made to 9 ml. The tube was then placed in a water bath at 38° and after attainment of temperature equilibrium the reaction was initiated by the addition of 1 ml. of enzyme solution. The pH of this solution was 9.6 as measured with a Beckman "high pH" glass electrode. After incubating for 30 minutes, proteins were precipitated with trichloroacetic acid and the liberated phosphate determined by the method of Fiske and Subbarow (10). Under these conditions the amount of inorganic phosphate formed was strictly proportional to the concentration of enzyme present.

Fig. 1 demonstrates the effect of varying concentrations of beryllium on the activity of alkaline phosphatase. It is apparent that beryllium in concentrations as low as 10^{-6} M causes appreciable inhibition of the enzyme. As the concentration of beryllium increases to a molarity of 10^{-5}, the inhibition reaches a maximum of 60.4 per cent. The phenomenon that maximal inhibition is reached at a relatively low concentration of beryllium is probably related to the low solubility of beryllium salts in alkaline solutions.

Table I demonstrates the effect on alkaline phosphatase of beryllium in combination with varying concentrations of magnesium. Fischer and Greep (11) have shown that the degree of activation of alkaline phosphatase by magnesium depends, among other factors, on the degree of purification and the age of the enzyme solutions. From Table I it can be calculated that activation of phosphatase by 0.01 M magnesium chloride in the absence of beryllium was 220 per cent, while with the enzyme which had been partly

---

[1] We wish to thank Mr. Bengt Kjellgren of the Brush Beryllium Company for this material.

inhibited by the addition of 10^{-5} M beryllium, an increase of only 120 per cent was caused by the addition of the same concentration of magnesium. Table I shows that the percentage inhibition by beryllium was greater when magnesium was present.   These findings indicate some interaction between the effects of magnesium and beryllium which might be interpreted as a competition of these two ions for the enzyme.   However, because
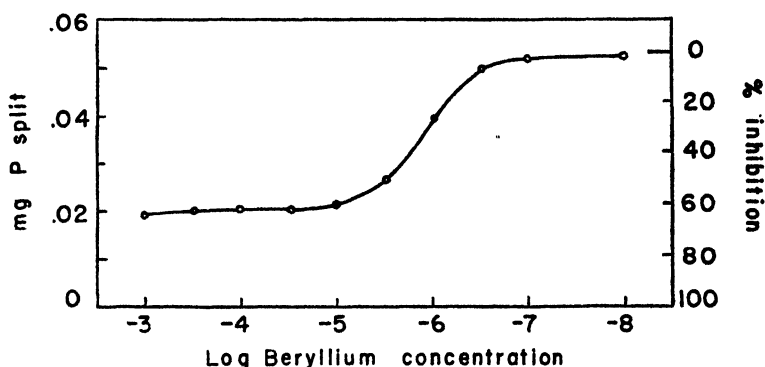
FIG. 1. Hydrolysis of sodium β-glycerophosphate by kidney phosphatase at pH 9.6 in presence of varying concentrations of beryllium.

<center>TABLE I</center>

Effect of Magnesium on Inhibition of Alkaline Phosphatase by Beryllium

Substrate, sodium β-glycerophosphate; pH 9.6; time of incubation, 30 minutes.

Concentration of Mg	Concentration of Be						
	0	10^{-6} M		3×10^{-6} M		10^{-5} M	
	P split	P split	Inhibition	P split	Inhibition	P split	Inhibition
mole per l.	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*
0	0.0425	0.0304	29	0.0235	45	0.0192	55
10^{-4}	0.112	0.0725	35	0.0462	59	0.0333	70
10^{-3}	0.136	0.0892	34	0.0542	60	0.0368	73
10^{-2}	0.136	0.0980	28	0.0642	53	0.0425	69

of the impurity of the enzyme preparation and because the mechanism of activation of phosphatase by magesium is not well understood (11, 12), such conclusions are not at present justified.

During the course of these experiments occasional phosphatase preparations were encountered which were activated by beryllium in concentrations of 10^{-7} and 10^{-8} mole per liter.   This activation, which was observed whether magnesium was present or not, was variable, occasionally reaching a maximum of 10 per cent, and was found only when enzyme solutions

which had aged in the refrigerator for some weeks were used. Hove, Elvehjem, and Hart (13) observed that dialyzed intestinal phosphatase was inhibited by zinc but was activated by it when amino acids were present. This observation suggested a possible similarity between the action of beryllium and that of zinc. The experiment represented in Table II was designed to ascertain whether glycine influenced the inhibition of phosphatase by beryllium. The data show that the activity of a freshly dialyzed enzyme was not altered by the addition of 1 mM glycine, regardless of the presence or absence of beryllium. At present we have no explanation for the occasional activation of certain phosphatase preparations by low concentrations of beryllium.

Beryllium hydroxide exhibits amphoteric properties and at the hydrogen ion concentration of the above experiments, a certain fraction of the metal exists as anion (14). If this beryllate were the ionic species which exerts

TABLE II

Inhibition of Alkaline Phosphatase by Beryllium with and without Glycine

Experimental conditions as given in Table I. The values are given in mg. of P liberated after 30 minutes.

Addition	Concentration of B					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	0
No glycine.......	0.0210	0.0230	0.0410	0.0506	0.0517	0.0514
Glycine, 10^{-3} M		0.0232	0.0432	0.0499	0.0510	0.0510

an effect on phosphatase, then a decrease of the hydrogen ion concentration, by increasing the acidic dissociation of the hydroxide, might effect an augmentation of the inhibition. If, on the other hand, the inhibition of phosphatase were due to beryllium ions (Be^{++}), a reverse effect might be expected. The experiment represented by Fig. 2 was designed to decide between these two possible mechanisms by testing the inhibiting effect of beryllium on phosphatase at varying hydrogen ion concentrations. The hydrogen ion concentration was altered by addition of hydrochloric acid or sodium hydroxide to the veronal buffer and the pH was measured with the glass electrode after addition of substrate and enzyme. Otherwise, conditions were exactly those of the preceding experiments. From Fig. 2 it would seem that the activity of the enzyme in the presence of beryllium increases with increasing hydrogen ion concentrations. If, however, the degree of hydrolysis is related to the value of the control containing no beryllium and expressed in terms of per cent inhibition, it becomes obvious that the strongest inhibition takes place near the hydrogen ion concentration of optimal enzyme activity. No conclusion can therefore be drawn as to the form in which beryllium is active as an inhibitor.

Table III gives the results of experiments in which various organic phosphate esters were incubated with alkaline phosphatase and magnesium salt
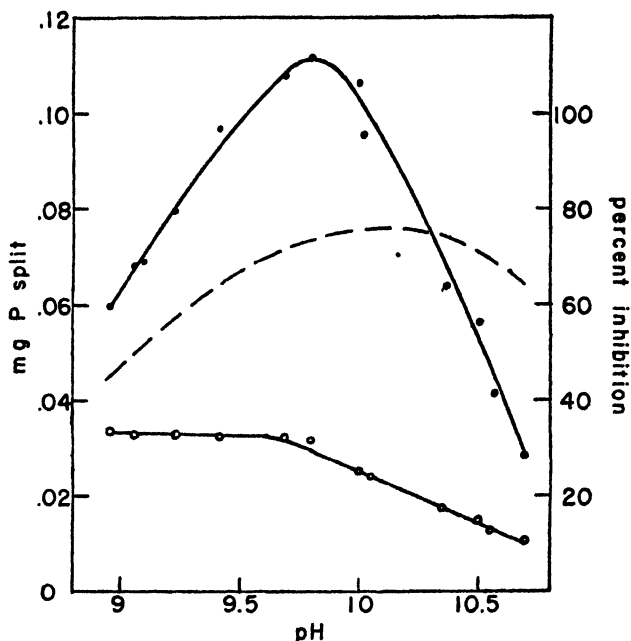
Fig. 2. The influence of hydrogen ion concentration on the hydrolysis of sodium β-glycerophosphate. ●, in the absence of beryllium; O, in presence of 10^{-5} M beryllium; dash line, per cent inhibition.

TABLE III

Effect of Beryllium on Alkaline Phosphatase in Presence of Various Substrates

Concentration of Mg^{++}, 10^{-3} M; time of incubation, 30 minutes.

Substrate		No Be	Be, 10^{-4} M		Be, 10^{-3} M	
		P split	P split	Inhibition	P split	Inhibition
	mM	mg.	mg.	per cent	mg.	per cent
β-Glycerophosphate	16.0	0.1563	0.0612	61		
Hexose diphosphate	0.24	0.0354	0.0159	55		
Phenyl phosphate	25.0	0.0486	0.0449	8	0.0256	47
" "	12.5	0.0729	0.0624	14	0.0326	55
" "	6.0	0.0985	0.0755	23	0.0371	62
" "	3.0	0.1209	0.0830	31	0.0443	63

in the presence of beryllium. Hexose diphosphate was added as a solution of its sodium salt which had previously been adjusted to pH 9.2. The amount of hydrolysis after 30 minutes was measured by determination of

inorganic phosphate as in the preceding experiments and a correction was applied for the amount of inorganic phosphate present as an impurity of the hexose diphosphate used. Phenyl phosphate was added as a solution of the commercial disodium salt (Eastman) adjusted to pH 9.2. The degree of hydrolysis was determined by phenol determination according to the procedure of King and Armstrong (15). For easy comparison the values in Table III are expressed in terms of phosphate. Table III shows that hydrolysis of all three substrates was inhibited by beryllium. In the case of phenyl phosphate it was found that the rate of hydrolysis decreased with increasing substrate concentrations. This finding is in disagreement with the values which Schmidt and Thannhauser (12) obtained with purified intestinal phosphatase. The degree of inhibition by beryllium increased markedly with decreasing substrate concentrations. It is evident

TABLE IV

Inhibition of Acid Phosphatase by Beryllium

Substrate, sodium β-glycerophosphate; buffer, 0.2 M acetate; pH 4.65; time of incubation, 60 minutes.

	Concentration of Be			
	10^{-3} M	10^{-4} M	10^{-5} M	0
P liberated, *mg*	0.0414	0.0480	0.0489	0.0489
Inhibition, %	15.3	1.8	0	

also that, in contrast to the hydrolysis of glycerophosphate (see Fig. 1), maximal inhibition was not reached at relatively low concentrations of beryllium. Inhibition increased when the beryllium concentration was raised from 10^{-4} M to 10^{-3} M and this relative increase became greater with rising substrate concentrations. These observations are easily explained by the assumption of chemical interaction between beryllium and phenyl phosphate, which would reduce the concentration of active beryllium below the solubility of its hydroxide.

The experiment given in Table IV demonstrates the effect of beryllium on acid phosphatase. Enzyme preparations and experimental conditions for the estimation of acid phosphatase activity were identical with those of the preceding experiments except that the reaction mixture was buffered at a pH of 4.65 with 0.2 M acetate buffer and the time of incubation was 60 minutes. Table IV shows that beryllium salt in concentrations up to 10^{-4} M was without effect on acid phosphatase and that even a concentration of 10^{-3} M caused only 15 per cent inhibition. This phenomenon is probably without biological significance and related to the known denaturing effect of higher concentrations of beryllium on proteins (16).

DISCUSSION

The experiments reported in this paper were conducted in an attempt to correlate the toxicity of beryllium with its possible action on certain well defined biochemical reactions. In addition to the osseous pathology mentioned in the introduction of this report, a variety of non-specific histological changes, principally involving parenchymatous tissues, have been ascribed to the administration of beryllium (for a complete discussion of the toxicology of beryllium, see (1)). Since phosphatase is a constituent of all these tissues, interference with the action of this enzyme could presumably explain such pathological changes. The fact that beryllium in very low concentrations has a strongly inhibitory action on alkaline phosphatase is an argument in favor of this explanation. It is still undecided, however, whether the inhibitory effect observed in experiments *in vitro* also obtains in living tissues.

SUMMARY

1. Alkaline kidney phosphatase is partly inhibited by beryllium in concentrations lower than 10^{-6} M.

2. Phosphatase activated by magnesium ions is inhibited by beryllium to a slightly greater degree than phosphatase not activated by the addition of magnesium.

3. The effect of beryllium on alkaline phosphatase is greatest in the region of the hydrogen ion activity of optimal enzyme activity.

4. The inhibition of alkaline phosphatase by beryllium is largely independent of the nature of the substrate. β-Glycerophosphate, hexose diphosphate, and phenyl phosphate were tested. In the case of the latter, the inhibition was found to decrease with increasing substrate concentration.

5. Acid phosphatase is not inhibited by beryllium in concentrations of 10^{-4} M. At a concentration of 10^{-3} M there is only 15 per cent inhibition.

BIBLIOGRAPHY

1. Sixth Saranac symposium of the Saranac Laboratory of the Trudeau Foundation, in press.
2. Guyatt, B. L., Kay, H. D., and Branion, H. D., *J. Nutr.*, **6**, 313 (1933).
3. Kay, H. D., and Skill, D. I., *Biochem. J.*, **28**, 1222 (1934).
4. Jacobson, S. A., *Arch. Path.*, **15**, 18 (1933).
5. Sobel, A. E., Goldfarb, A. R., and Kramer, B., *J. Biol. Chem.*, **108**, 395 (1935).
6. Gardner, L. U., and Heslington, H. F., *Federation Proc.*, **5**, 221 (1946).
7. Cloetens, R., *Biochem. Z.*, **310**, 42 (1941).
8. Greenstein, J. P., Carter, C. E., Chalkley, H., and Leuthardt, F. M., *J. Nat. Cancer Inst.*, **7**, 9 (1945).
9. Bodansky, A., *J. Biol. Chem.*, **101**, 93 (1933).
10. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).

11. Fischer, C. J., and Greep, R. O., *Arch. Biochem.*, **16**, 199 (1948).
12. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **149**, 369 (1943).
13. Hove, E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **134**, 425 (1940).
14. Mellor, J. W., A comprehensive treatise on inorganic and theoretical chemistry, London, **4**, 226 (1946).
15. King, E. J., and Armstrong, A. R., *Canad. Med. Assn. J.*, **31**, 376 (1934).
16. Hyslop, F., Palmes, E. D., Alford, W. C., Monaco, A. R., and Fairhall, L. T., *Nat. Inst. Health Bull., No. 181* (1943)

OBSERVATIONS ON THE EFFECTS OF BERYLLIUM
ON ALKALINE PHOSPHATASE*

By ROBERT S. GRIER, MARGARET B. HOOD, and MAHLON B. HOAGLAND†

(From the Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital, Boston)

In the past 5 years a number of papers have dealt with the clinical aspects of exposure to various beryllium compounds (1–4). Because of these reports several laboratories (5) have attempted to reproduce in animals the lesions seen in the human cases but with only partial success. In 1946, Dr. Leroy Gardner (6) noted that the intravenous injection of zinc-beryllium silicate or beryllium oxide into rabbits resulted in the development of osteogenic sarcomas after a latent period as short as $5\frac{1}{2}$ months or as long as 1 year. This observation has been confirmed in this laboratory and will be described in a later paper.

Several authors (7–10) have reported that the addition of beryllium carbonate to the diet of young rats led to the development of rachitic changes which have been termed "beryllium rickets." Extremely low levels of serum phosphate and a marked lowering of the alkaline phosphatase content of the kidney accompanied the development of this lesion. Furthermore, this lesion could not be prevented by cod liver oil or irradiated ergosterol, though Kay and Skill (10) were able to prevent it by the daily parenteral administration of glycerophosphate. These authors think that the lesion, at least in part, is due to the precipitation of beryllium phosphate in the intestine, making the phosphate unavailable for absorption. However, Sobel, Goldfarb, and Kramer (11) in studying *in vitro* the calcifying power of ordinary rachitic bone and beryllium rachitic bone in normal serum noted a markedly diminished calcifying power in the latter compared to the former. They also noted that, although the addition of viosterol to the beryllium rachitogenic diet produced a rise in the calcium and phosphorus product in the serum, the rickets was not prevented and the defect in the calcifying power *in vitro* was still present. This work suggested that in addition to the factor of poor absorption of phosphate from the intestine there was also a local factor which interfered with calcification. In 1943, Hyslop *et al.* (12) presented evidence that beryllium tends to be stored in bone.

Because of this previous work and because of the well known high phosphatase levels which are found in osteogenic sarcoma, Paget's disease, rickets, and osteoblastic bone lesions, it seemed worth while to study the effects of beryllium on alkaline phosphatase *in vitro* in the hope of shedding some light on the mechanism of beryllium toxicity.

There are a few references (13) to the effects of beryllium on alkaline phosphatase *in vitro*, but the results have been variable and experimental data not given, and apparently no extensive study has been made.

Methods

Enzyme Preparations Used—At the outset of this study crude enzyme preparations were made according to the method of Franseen and McLean (14) from bone, kidney, and intestine of freshly decapitated Wistar rats. These extracts were dialyzed in cellophane bags for 48 hours against frequent changes of distilled water at 7°. At the end of this period the crude extracts were nearly free of inorganic phosphate. Extracts were also used from human serum with high phosphatase activity and radium-induced osteogenic sarcomas from the rat.

Later in the study, through the courtesy of Dr. Gerhard Schmidt, we were able to secure some highly purified intestinal phosphatase prepared by him according to the method he has previously described (15).

Determination of Phosphatase Activity—The phosphatase activity was determined in a manner similar to that described by Franseen and McLean (14) with minor modifications used through experience in this laboratory.[1] All reactions were incubated for a period of 30 minutes at a temperature of 37° and at pH 9.0, and the liberated phosphate was determined by the method of Fiske and Subbarow (16) as modified for use in the Evelyn photoelectric colorimeter (17). The substrate used in these experiments was sodium β-glycerophosphate (Eastman), the concentration of which was 0.015 M in the incubation mixture in all experiments, and the amount of substrate splitting never exceeded 5 per cent. The concentration of glycine (as buffer) was 0.06 M. All determinations were done in duplicate.

The beryllium solutions were prepared from $BeSO_4 \cdot 4H_2O$ (c.p., Eimer and Amend) and a stock standard solution was made in the concentration of 1 mg. of Be ion = 1.0 cc. The magnesium solutions were made from $MgSO_4 \cdot 7H_2O$ (c.p., Merck, Blue Label) and the citrate solutions from sodium citrate $\cdot 2H_2O$ (Merck, Blue Label). All concentrations are expressed throughout this paper as moles per liter of incubation mixture.

Results

Effects of Varying Beryllium Concentration on Alkaline Phosphatase— Studies of the effect of varying concentration of beryllium on the alkaline

phosphatase activity of undialyzed and dialyzed extracts and of a concentrated intestinal phosphatase were made. The sources of these have been described above. In order to make the results as nearly comparable as possible, the dialyzed extracts were so diluted that the controls would split between 0.02 and 0.04 mg. of phosphorus in 30 minutes of incubation. The concentrations of beryllium used varied through the range of 6×10^{-7} to 2.4×10^{-4} M in the incubation tube. Because of the amphoteric properties of beryllium and the alkaline pH of the reaction, it seemed probable that beryllium existed as the beryllate ion (BeO^-) rather than as the beryllium

TABLE I

Effect of Varying Concentrations of Beryllium on Rat Osteogenic Sarcoma, Rat Bone, Rat Intestinal Phosphatase, and Human Serum

Be concentration per liter incubation mixture	Rat sarcoma extract		Rat bone extract		Rat intestinal extract (undialyzed)		Be concentration per liter incubation mixture	Human serum (undialysed)	
	P split	Inhibition	P split	Inhibition	P split	Inhibition		P split	Inhibition
30 min. incubation, pH 9.0							60 min. incubation, pH 9.0		
M	mg	per cent	mg.	per cent	mg.	per cent	M	mg.	per cent
0	0.052	0	0.039	0	0.027	0	0	0.034	0
1.2×10^{-6}	0.051	1					2.4×10^{-6}	0.033	1
2.4×10^{-6}					0.023	15	6.0×10^{-6}	0.030	11
6.0×10^{-6}	0.044	15	0.032	18	0.018	34	1.2×10^{-5}	0.027	19
1.2×10^{-5}	0.041	22	0.033	16	0.015	44	6.0×10^{-5}	0.020	39
2.4×10^{-5}	0.037	28	0.030	23	0.015	46	1.2×10^{-4}	0.019	42
3.6×10^{-5}			0.030	22	0.012	55			
6.0×10^{-5}			0.027	31	0.015	42			
8.4×10^{-5}			0.027	30	0.010	61			
1.2×10^{-4}			0.024	39	0.011	60			
2.4×10^{-4}			0.024	39					

ion (Be^{++}). At concentrations around 10^{-4} M a white, flocculent precipitate tended to separate at the end of incubation, especially with undialyzed preparations, which contained a considerable amount of inorganic phosphate. Presumably this precipitate was beryllium phosphate, which is highly insoluble in alkaline media. Precipitation was not noted as concentrations of beryllium lower than 2.4×10^{-4} M. We have demonstrated, however, that all phosphate liberated in the incubation, whether a precipitate is seen or not, is determined in the phosphate estimation. The addition of the trichloroacetic acid prior to filtering the incubation mixture redissolves any insoluble beryllium phosphate present.

The results of these experiments are presented in Tables I and II. It is apparent from these data that beryllium exerts a definite inhibitory effect on alkaline phosphatase activity in concentrations as low as 10^{-7} M. It is

interesting to note that extracts of bone, kidney, human serum, and rat osteogenic sarcoma show approximately the same degree of inhibition at the maximal beryllium concentration of 1.2×10^{-4} M, namely about 40 per cent. In contrast, extracts of intestinal phosphatase, both dialyzed and undialyzed, as well as the concentrated extract are inhibited 60 to 70 per cent at the same concentration of beryllium. The lack of increasing inhibitory effect of beryllium in concentrations above 1.2×10^{-4} M is probably due to the precipitation of beryllium phosphate, thus limiting the effective concentration of beryllium.

Effect on Intestinal Phosphatase of Varying Magnesium Concentration in Presence of Constant Beryllium Concentration—As a number of investigators

TABLE II

Effect of Varying Beryllium Concentrations on Dialyzed Rat Intestine, Kidney, Bone, and Concentrated Intestinal Phosphatase

30 minutes incubation, pH 9.0.

Be concentration per liter	Intestine		Kidney		Bone		Concentrated intestinal phosphatase (1:000)		
	P split	Inhibition	P split	Inhibition	P split	Inhibition	Be concentration per liter	P split	Inhibition
M	mg.	per cent	mg.	per cent	mg.	per cent	M	mg.	per cent
0	0.033	0	0.028	0	0.016	0	0	0.024	0
2.4×10^{-6}	0.024	26	0.024	15	0.015	7	6×10^{-7}	0.023	8
6.0×10^{-6}	0.018	45	0.023	19	0.015	5	1.2×10^{-6}	0.022	12
1.2×10^{-5}	0.014	58	0.019	32	0.013	21	2.4×10^{-6}	0.018	20
6.0×10^{-5}	0.010	69	0.016	42	0.010	35	6.0×10^{-6}	0.015	41
1.2×10^{-4}	0.010	69	0.017	41	0.010	37	1.2×10^{-5}	0.011	55
							6.0×10^{-5}	0.008	67
							1.2×10^{-4}	0.007	71

have previously reported that magnesium is an activator of dialyzed alkaline phosphatase preparations, this experiment was designed to determine whether the inhibition by beryllium might be reversed by the addition of magnesium. Beryllium in a concentration of 1×10^{-4} M was added to the substrate and magnesium, in varying concentrations, was added prior to the introduction of the enzyme. As a control, magnesium was added to a substrate which contained no beryllium. The data in Tables III and IV indicate that the optimal concentration of magnesium is about 0.003 M in the absence of beryllium, approximately the value noted by Jenner and Kay (13). Although magnesium activates both reactions, the per cent of inhibition by beryllium is seen not to be appreciably influenced by the presence of magnesium. These observations suggest a competitive relationship of beryllium and magnesium to the enzyme molecule.

Effect of Storing Enzyme with Magnesium or Beryllium for 18 Hours Prior to Incubation—The preceding experiments suggested that the relative

affinity of the metals under consideration for the enzyme might be further elucidated by varying the time prior to incubation during which they are allowed contact with the enzyme. Thus, beryllium in a concentration of 4×10^{-5} M and magnesium in a concentration of 3×10^{-3} M were mixed

TABLE III

Effect of Adding Increasing Amounts of Magnesium to Dialyzed Rat Intestinal Phosphatase in Presence and Absence of Beryllium

30 minutes incubation, pH 9.0; three different intestinal extracts.

Experiment No.	Mg concentration per liter	No Be P split	Be 1×10^{-4} M P split	Inhibition
	M	*mg.*	*mg.*	*per cent*
I	0	0.031	0.007	77
	5×10^{-5}	0.033	0.007	79
	1×10^{-3}	0.046	0.012	73
	2×10^{-3}	0.055	0.013	76
II	0	0.028	0.007	77
	2×10^{-3}	0.043	0.013	71
	1×10^{-2}	0.042	0.013	70
	4×10^{-2}	0.049	0.013	74
III	0	0.038	0.008	78
	2×10^{-3}	0.062	0.011	83
	1×10^{-2}	0.055	0.011	81
	4×10^{-2}	0.056	0.009	83
	1.6×10^{-1}	0.088	0.010	88
	3.2×10^{-1}	0.098	0.012	88

TABLE IV

Effects of Adding Increasing Amounts of Magnesium to Concentrated Intestinal Phosphatase in Presence and Absence of Beryllium

30 minutes incubation, pH 9.0.

Mg concentration per liter	No Be P split	Be, 1×10^{-4} M P split	Inhibition
M	*mg.*	*mg.*	*per cent*
0	0.029	0.007	75
3×10^{-3}	0.040	0.012	71
3×10^{-2}	0.031	0.011	65
3×10^{-1}	0.039	0.011	73

with a purified intestinal phosphatase preparation, singly and in combination, and were stored at 7° for 18 hours before incubation with the substrate. The results of such an experiment are shown in Fig. 1. The activating effect of magnesium and the inhibitory effect of beryllium, when each is present alone, are not appreciably enhanced by standing with the

enzyme for 18 hours.   When both metals are allowed to stand with the enzyme for 18 hours, the effect is the same as if beryllium were present alone.   Even when magnesium is stored with the enzyme alone and beryllium is only added just prior to incubation, the inhibitory effect of the latter predominates.   This series of experiments lends further credence to the concept that the relationship of beryllium and magnesium is competitive in relation to this enzyme, and that the influence of beryllium is somewhat dominant.
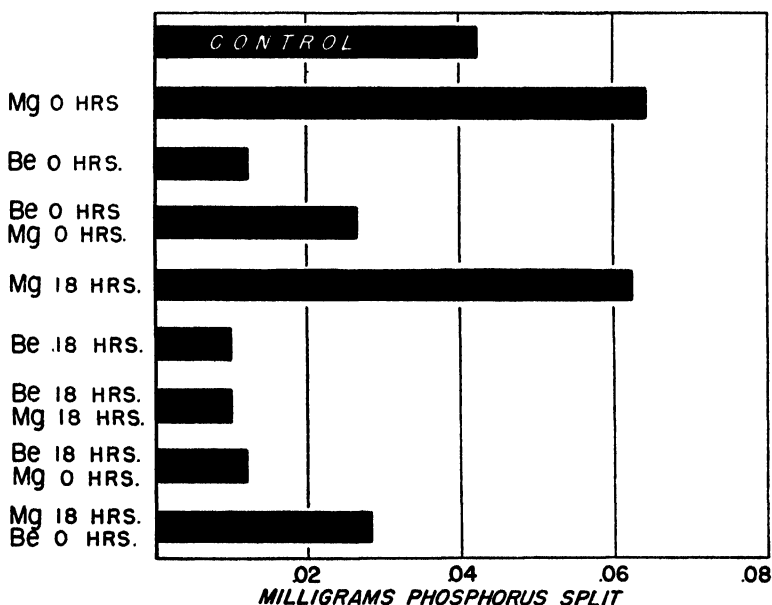
Fig. 1. Effect of allowing beryllium or magnesium to stand with the enzyme for 18 hours prior to incubation.   "Hours" refers to the time of contact of the element with the enzyme before incubation.

Effect of Addition of Magnesium after Beryllium Has Been in Contact with Enzyme for Varying Periods of Time—The conditions of the experiment were the same as the preceding one as far as beryllium and magnesium concentrations and enzyme preparations were concerned.   Beryllium was stored with the enzyme for increasing lengths of time before incubation was initiated, magnesium being added at the start of incubation.   The results of this experiment appear in Fig. 2.   The maximal inhibitory effect of beryllium is attained within 4 hours, while the ability of magnesium to activate the enzyme, though not completely lost, is reduced to a very low and constant value after beryllium has been in contact with the enzyme for 9 hours.

Effect of Sodium Citrate on Beryllium Inhibition and Magnesium Activation—The facility with which the citrate ion forms metal complexes is well known and wide use is made of this property in analytical chemistry (18). In medicine, citrate has been employed to mobilize heavy metals such as lead through the formation of soluble citrate complexes. These facts engendered the idea that perhaps the inhibition of phosphatase by beryllium might be prevented by the introduction of citrate, to form a beryllium-citrate complex.
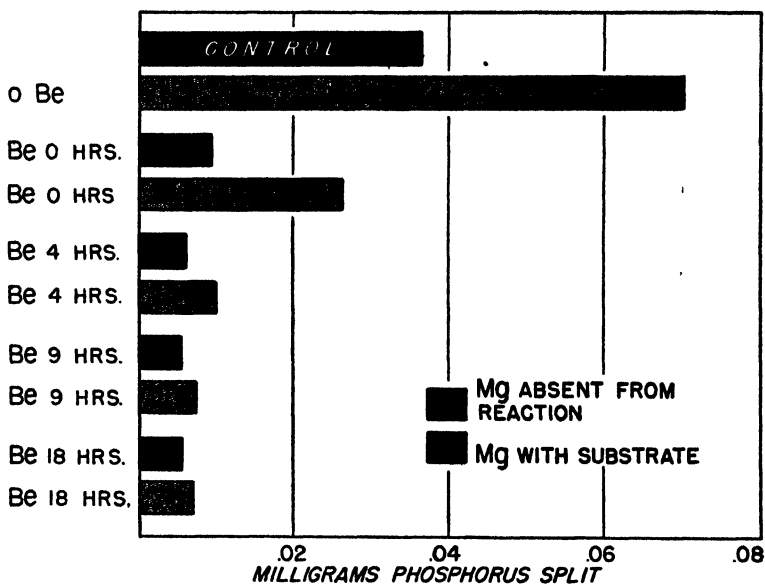
Fig. 2. Effect of varying the length of time prior to incubation during which beryllium is in contact with the enzyme.

Jenner and Kay (13) mention that citrate is an inhibitor of alkaline phosphatase but give no experimental data on the concentrations employed. Gutman and Gutman (19) have noted that citrate ion in concentrations greater than 0.04 M interferes with color development in the phosphate determination. By using standard phosphate solutions with and without varying concentrations of citrate (from 1×10^{-4} to 2×10^{-2} M) in the presence of substrate, we were able to show that at the highest citrate concentrations the variation between citrated and non-citrated standards was less that 6 per cent. Experiments in which the enzyme was used with and without citrate over the same concentration range showed no significant difference. On the basis of these observations it was felt that any observed effect of citrate on beryllium inhibition or magnesium activation of phos-

phatase would not be due to any defect in color development or to a direct effect of citrate on the enzyme.

In these experiments magnesium or beryllium was added to the substrate and varying concentrations of citrate (as sodium citrate) added prior to the introduction of the enzyme. The results of such an experiment are recorded in Fig. 3.

It can be seen that with increasing concentrations of citrate in the reaction mixture the activating effect of magnesium is reduced toward the
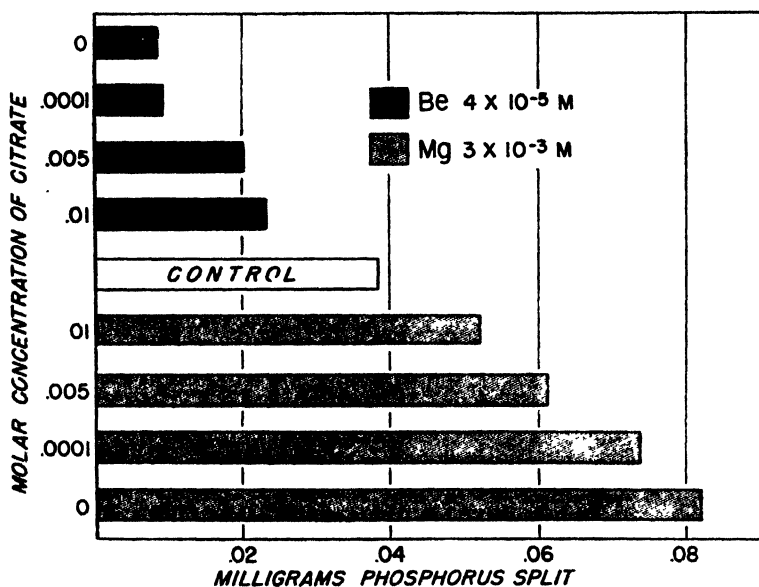
FIG. 3. Effect of increasing concentrations of citrate on beryllium inhibition and magnesium activation of phosphatase.

region of the control. Similarly, the inhibitory influence of beryllium is considerably reduced. In one experiment, magnesium activation was completely abolished by 0.02 M citrate, a concentration which did not completely erase the beryllium effect. When both beryllium and magnesium were added together with 0.02 M citrate, neither was able to exert its characteristic effect.

Effects of Aging and Dilution of Concentrated Intestinal Phosphatase on Phenomena Described Above—We have found that there is a gradual change in the behavior of the concentrated intestinal phosphatase that we have been using for these experiments. Over a period of about a month the activity is about halved, while the activating effect of magnesium increases from 40 to 300 per cent. On the other hand, the percentage of inhibition

by beryllium remains almost constant throughout this period. It is noted that with the passage of time the characteristic dominance of beryllium over magnesium is lost. Beryllium dominance can be brought back, however, if a preparation is used which has been allowed to stand in a diluted state for a day or two before use, thus reducing the magnesium-activating effect. The findings in reference to changes in activity and magnesium effect in aging of the enzyme are in accord with those of Fischer and Greep (20). The explanation of these changes is obscure.

<div align="center">DISCUSSION</div>

Evidence has been presented to show that beryllium exercises an inhibitory effect on the activity of alkaline phosphatase from various sources. The experiments suggest that beryllium as inhibitor and magnesium as activator compete for control of the enzyme molecule. Under the conditions of the experiments, which were such as to allow each metal to act in its optimal concentration and involved the use of relatively fresh preparations, beryllium seemed to have a greater affinity for the enzyme than did magnesium. It should be mentioned that the use of other substrates (phenyl phosphate and phenolphthalein phosphate) did not alter these findings. Furthermore, the use of a veronal buffer in the hands of another investigator gave the same results.[2]

It is suggested that the observed effect of citrate on beryllium inhibition of phosphatase might have some value in the treatment of beryllium poisoning.

These findings are difficult to interpret in relation to the sarcomagenic and rachitogenic properties of beryllium, and the clarification of the intimate relationship of these metals to the enzyme must await further advances in the purification of the latter. There is no clear cut evidence that the toxicity of beryllium is related to its effects on phosphatase, but it is felt that these investigations shed some light on the biochemistry of this element and may lead to further clarification of the toxicology of beryllium as well as the biochemistry of sarcomagenesis.

<div align="center">SUMMARY</div>

1. Crude alkaline phosphatase preparations from several sources and a relatively pure alkaline phosphatase are inhibited by small concentrations of beryllium.

2. The per cent of inhibition of phosphatase by beryllium is not appreciably affected by the presence of magnesium.

3. The inhibitory effect of beryllium is more marked than the activating effect of magnesium.

[2] Klemperer, F., personal communication.

4. The inhibitory effect of beryllium reaches a maximum in less than 4 hours. The activating effect of magnesium in the presence of beryllium is almost completely lost when beryllium has been in contact with the enzyme for 9 hours.

5. Citrate reverses the beryllium inhibition and the magnesium activation.

6. It is suggested that there is a competitive relationship between beryllium and magnesium to the enzyme molecule, which is of interest because of the known rachitogenic and sarcomagenic effects of beryllium.

We are indebted to Dr. Joseph C. Aub, Dr. Paul C. Zamecnik, Miss Dorothy M. Tibbetts, and Dr. F. Klemperer for useful suggestions during the course of this study, and to Dr. G. Schmidt for supplying some of his enzyme.

BIBLIOGRAPHY

1. Van Ordstrand, H. S., Hughes, R., DeNardi, J. M., and Carmody, M. G., *J. Am. Med. Assn.*, **129**, 1080 (1945).
2. Van Ordstrand, H. S., Hughes, R., DeNardi, J. M., and Carmody, M. G., *Cleveland Clin. Quart.*, **10**, 10 (1943).
3. Hardy, H. L., and Tabershaw, I. R., *J. Ind. Hyg. and Toxicol.*, **28**, 197 (1946).
4. Martland, H. S., Brodkin, H. A., and Martland, H. S., Jr., *J. Med. Soc. New Jersey*, **45**, 5 (1948).
5. Sixth Saranac symposium of the Saranac Laboratory of the Trudeau Foundation, in press.
6. Gardner, L. U., *Federation Proc.*, **5**, 221 (1946).
7. Guyatt, B. L., Kay, H. D., and Branion, H. D., *J. Nutr.*, **6**, 313 (1934).
8. Jacobsen, S. B., *Arch. Path.*, **15**, 18 (1933).
9. Jones, J. H., *Am. J. Physiol.*, **124**, 230 (1938).
10. Kay, H. D., and Skill, D. I., *Biochem. J.*, **28**, 1222 (1934).
11. Sobel, A. E., Goldfarb, A. R., and Kramer, B., *J. Biol. Chem.*, **108**, 395 (1935).
12. Hyslop, F., Palmes, E. D., Alford, W. C., Monaco, A. R., and Fairhall, L. T., *Nat. Inst. Health Bull.*, *No. 181* (1943).
13. Jenner, H. D., and Kay, H. D., *J. Biol. Chem.*, **93**, 733 (1931).
14. Franseen, C. C., and McLean, R., *Am. J. Cancer*, **24**, 299 (1935).
15. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **149**, 369 (1943).
16. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
17. Folin, O., Laboratory manual of biological chemistry, New York and London, 5th edition, 341 (1934).
18. Fales, H. A., and Kenny, F., Inorganic quantitative analysis, New York (1943).
19. Gutman, E. B., and Gutman, A. B., *J. Biol. Chem.*, **136**, 201 (1940).
20. Fischer, C. J., and Greep, R. O., *Arch. Biochem.*, **16**, 199 (1948).

THE PREPARATION OF C¹⁴-LABELED BIOTIN AND A STUDY OF ITS STABILITY DURING CARBON DIOXIDE FIXATION

By DONALD B. MELVILLE, JOHN G. PIERCE,* and C. W. H. PARTRIDGE

(*From the Department of Biochemistry, Cornell University Medical College, New York City*)

Several lines of evidence indicate that biotin is involved in the biological fixation of carbon dioxide. Lardy, Potter, and Elvehjem (1) have demonstrated that the biotin requirement of· *Lactobacillus arabinosus* is partially replaceable by oxalacetate and that the growth stimulation of this organism by carbon dioxide is evident only when biotin is present. From microbiological studies with antimetabolites, Shive and Rogers (2) have concluded that biotin is effective in the formation of α-ketoglutarate in *Escherichia coli*, and in the formation of oxalacetate in *L. arabinosus*. Lichstein and Umbreit (3) have suggested that biotin is a coenzyme in the oxalacetate decarboxylase enzyme system in *E. coli*. Ochoa and his coworkers (4) found a decreased activity of the carbon dioxide-fixing "malic" enzyme in livers from biotin-deficient turkeys, whereas livers from folic acid-deficient turkeys were normal in this respect. On the other hand, these workers were unable to identify biotin as a constituent of the purified enzyme. In biotin-deficient rats, Robertson and Lardy (5) have reported a decreased fixation of labeled carbon dioxide in aspartic acid and arginine.

While the above studies implicate biotin as an important factor in carbon dioxide fixation, the mechanism involved remains obscure. Based on the structure of biotin and its known chemical reactions, it was early postulated (6) that the vitamin might enter into biological carbon dioxide-transferring mechanisms by virtue of an opening and closing of the ureido ring system. The carbon dioxide thus incorporated into the biotin molecule would then presumably be transferred to pyruvic acid or other substrate concerned in the fixation of carbon dioxide.

$$
\begin{array}{ccc}
\begin{array}{ccc}
NH_2 & & NH_2 \\
| & & | \\
CH & \!\!\!-\!\!\!- & CH \\
| & & | \\
CH_2 & & CH(CH_2)_4COOH \\
& \diagdown \; \diagup & \\
& S &
\end{array}
& \underset{\rightleftharpoons}{\overset{CO_2}{\longrightarrow}} &
\begin{array}{ccc}
& \overset{\displaystyle CO}{\diagup \; \diagdown} & \\
NH & & NH \\
| & & | \\
CH & \!\!\!-\!\!\!- & CH \\
| & & | \\
CH_2 & & CH(CH_2)_4COOH \\
& \diagdown \; \diagup & \\
& S &
\end{array}
\end{array}
$$

---
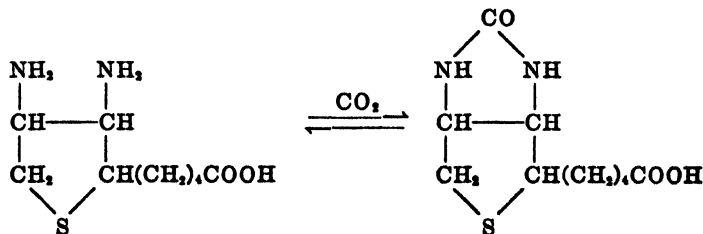
* Postdoctorate Fellow of the American Chemical Society.

Such a mechanism is amenable to investigation with radioactive carbon. Demonstration that the ureido carbon of biotin is biologically labile would offer strong evidence for the mode of action of biotin in carbon dioxide fixation. Conversely, demonstration of its stability would afford a convenient method of labeling biotin with isotopic carbon for further investigations of its metabolic rôle. Accordingly, we have synthesized biotin containing C^{14} in the ureido carbon atom, and have studied its fate during carbon dioxide fixation in *L. arabinosus*.

The synthesis of the C^{14}-labeled biotin was accomplished by utilization of the previously described reaction of phosgene with the diaminocarboxylic acid derived from biotin (7). Radioactive phosgene was prepared from C^{14}-containing carbon monoxide, which in turn was conveniently prepared by the dehydration of radioactive formic acid obtained by the reduction of bicarbonate (8). Because of the relatively low requirements of living cells for biotin, the radioactive biotin was synthesized with the highest possible specific activity of C^{14} in the ureido carbon atom.

In a preliminary experiment with the C^{14}-labeled biotin, aspartic acid was isolated from *L. arabinosus* cells which had been grown in an aspartic acid-free medium in the presence of the labeled vitamin, since much of the carbon dioxide fixed by this microorganism appears in the synthesized aspartic acid (9). No detectable radioactivity was present in the isolated aspartic acid or in the gas phase above the culture.

Because of the extremely high dilution of radioactivity inherent in this type of experiment if transfer of C^{14} did occur, a different approach was devised to eliminate the necessity of detecting radioactivity of a low order. It was also desirable to ascertain whether C^{14} might be transferred to substances other than aspartic acid.

As described in the experimental section of this paper, *L. arabinosus* cells were grown in an aspartic acid-free medium in the presence of a known amount of radiobiotin. The cells and medium were then separated and the amount of radiobiotin in each was determined by an isotope dilution technique. In the case of the medium, this was accomplished simply by the addition of a conveniently large amount of non-isotopic biotin to the medium and reisolation of the biotin and purification to constant specific radioactivity. In the case of the cells, non-isotopic biotin was added prior to acid hydrolysis and reisolated from the hydrolyzed cells.

Quantitative estimation of the radioactivity present in the isolated biotin samples was accomplished by comparison in the Geiger-Müller counter with a radiobiotin standard prepared by dilution of the highly active radiobiotin with non-isotopic biotin. This procedure eliminated the necessity of conversion of the samples to barium carbonate, with the resultant decrease in specific activity.

Results

The results obtained are given in Table I, in terms of total counts and also, by calculation from the radioactivities, in terms of biotin. For comparison, Table I also lists the biotin contents of the cells and medium obtained by microbiological assay with *L. arabinosus* and with *Saccharomyces cerevisiae*.

It can be seen that 93 per cent of the C^{14} originally present in the radiobiotin is recovered at the end of the growth period as radiobiotin. Furthermore, the *L. arabinosus* assay values for the biotin contents of the cells and medium are in good agreement with the biotin contents calculated from the radioactivity data. These results demonstrate that no appreciable exchange of the C^{14}-labeled ureido carbon atom of biotin with

TABLE I

Radiobiotin Content and Microbiological Assay of Medium and Cells after Growth of L. arabinosus in Presence of 7.32 γ of Radiobiotin

	Radiobiotin analyses		L arabinosus	S cerevisiae
	counts per min.	*γ radiobiotin**	*γ biotin*	*γ biotin*
Cells	1955	1.36	1.32	1.8
Medium	7850	5.48	5.53	8.9
Total	9805	6.84	6.85	10.7

* Calculated from comparison with the standard radiobiotin preparation containing 3 66 γ of radiobiotin and possessing 5250 counts per minute.

non-isotopic carbon has occurred. The conditions of the growth experiment are such that carbon dioxide fixation involving biotin must be actively occurring, since in the absence of aspartic acid in the medium *L. arabinosus* synthesizes the aspartic acid needed for its growth from oxalacetate, which in turn is formed by biotin-controlled carbon dioxide fixation (10, 1). That fixation was indeed occurring under the conditions of the present experiment was confirmed by the demonstration of the formation of C^{14}-labeled aspartic acid by the cells of a control culture grown under similar conditions, with non-labeled biotin and in the presence of radioactive carbon dioxide.

It seems logical to expect that during active growth an appreciable fraction of the cellular biotin would be participating in the enzymatic reactions concerned with carbon dioxide fixation and that consequently a readily detectable portion of the C^{14} of the radiobiotin should have been replaced with non-radioactive carbon if the mechanism under investigation were correct. The results obtained, therefore, are a strong indication that the ureido carbon atom of biotin is not directly involved in the

transfer of carbon dioxide during carbon dioxide fixation in *L. arabinosus*. Studies on biotin-deficient rats injected with radiobiotin suggest that essentially the same conclusion with regard to biotin and carbon dioxide fixation can be drawn for the mammal (unpublished observations).

The assays with *S. cerevisiae* demonstrate that a considerable amount of *L. arabinosus*-inactive, yeast-active material is produced by *L. arabinosus* during growth. The major portion (85 per cent) of this material is present in the medium at the end of the growth period. If it is derived exclusively or in large part from biotin, it must possess a biological activity markedly higher than that of biotin, in view of the high recovery of radiobiotin. That it is not readily converted to biotin was shown by the fact that *L. arabinosus* assay values were not increased after hydrolysis of the medium.

<div align="center">EXPERIMENTAL</div>

Synthesis of C^{14}-Labeled Biotin—The radioactive barium carbonate used as the starting material was purchased from the Clinton Laboratories of the Monsanto Chemical Company, on allocation from the United States Atomic Energy Commission. Approximately 1 mc. (101.2 mg.) was converted to potassium formate by the procedure previously described (8). The formate solution was evaporated to dryness in a 20 × 200 mm. glass tube equipped with a ground glass joint. A glass cup containing 2 ml. of concentrated sulfuric acid was placed in the tube and the tube was closed by means of a head equipped with suitable inlet and outlet tubes which could be sealed by means of stop-cocks. After evacuation of the system with an oil pump, the acid and formate were mixed by tipping the apparatus, and the mixture was warmed to 70° for a few minutes to complete the liberation of carbon monoxide. A slight excess (12 ml.) of dry chlorine gas was drawn into the system from a gas burette, and the gas mixture was irradiated with a 100 watt internal reflector lamp for 30 minutes at a distance of 12 inches.

50 mg. of the diaminocarboxylic acid sulfate derived from *d*-biotin (11) and 1.5 ml. of 1.7 N NaOH solution were placed in a second, similar glass apparatus. The inlet tube of this system was attached to a tube containing a small amount of mercuric sulfide, for the removal of unchanged chlorine. The apparatus was flushed with nitrogen and was connected to the apparatus containing the phosgene. A stream of nitrogen was used to carry the phosgene through the tube of mercuric sulfide into the vessel containing the diamino acid, where it was frozen out by means of a liquid nitrogen bath. After 1.5 hours, the flow of nitrogen was stopped and the vessel containing the frozen phosgene and diamino acid was evacuated briefly by the use of an oil pump, and was sealed by means of

stop-cocks. After the contents had warmed to room temperature the apparatus was shaken for 1 hour. The solution was then acidified and the liberated carbon dioxide was collected in a scrubber containing alkali. The d-biotin crystals which separated from the acidified solution were collected and recrystallized from water. The yield was 10.4 mg., micro melting point 226–230°. This material was used in the growth experiments.

The mother liquors from the first crystallization were made alkaline and treated with non-isotopic phosgene in excess. From this reaction 25 mg. of biotin possessing a lower order of radioactivity were obtained.

For radioactivity analyses, samples of the biotin were oxidized to carbon dioxide by the wet combustion method of Van Slyke and Folch (12). Analyses were carried out on barium carbonate pads as previously described (8). From a total of 1.27×10^8 counts per minute in the barium carbonate used as starting material, 1.28×10^7 counts per minute were obtained in the 10.4 mg. of biotin, and 5.74×10^6 counts per minute in the 25 mg. sample of biotin obtained from the mother liquors. The carbon dioxide recovered from the last step of the synthesis contained 7.1×10^7 counts per minute. Therefore approximately 70 per cent of the original radioactivity was accounted for in these fractions. No attempt was made in this work to improve the yield in the last step of the synthesis.

Growth of L. arabinosus with Radiobiotin—A culture of *L. arabinosus* 17-5 which had been carried in dextrose-yeast-agar stab cultures (13) was grown for 20 hours at 37° in 10 ml. of basal medium (14) containing 0.0002 γ of biotin. The cells were separated by centrifugation, washed with 10 ml. of saline, and resuspended in 20 ml. of saline. Two 450 ml. portions of aspartic acid-free medium (15), each containing 3.66 γ of the radioactive biotin, were sterilized in 3 liter Fernbach flasks and then inoculated with 1 ml. portions of the saline suspension. The flasks were incubated for 28 hours at 37°.

At the end of the growth period, the cells were separated by centrifugation, washed three times with cold water, and dried from the frozen state. The weight of the dried cells was 327 mg., and the volume of the combined medium and washings was 960 ml. A control culture without added radiobiotin showed no growth.

Microbiological assays on the medium and cells were carried out by the *L. arabinosus* method of Snell and Wright (13) and the yeast method of Snell, Eakin, and Williams (16), with modifications in the media (14). For assay the cells were hydrolyzed with 2 N sulfuric acid for 2 hours. The results are given in Table I. Hydrolysis of the medium under the same conditions did not increase the biotin values over those obtained with unhydrolyzed medium.

Isolation of Radiobiotin from Medium—To 500 ml. of medium were added 150 mg. of non-isotopic *d*-biotin (Merck), which was dissolved by warming. The biotin was reisolated by adsorption on 5 gm. of charcoal and elution from the charcoal with two 50 ml. portions of a 1:6:3 concentrated ammonia-ethanol-water mixture. The eluate was evaporated to dryness and the residue was dissolved in a small volume of water. Acidification with HCl yielded crystals of biotin. For further purification the biotin was converted to the methyl ester by treatment with diazomethane, and the ester was sublimed *in vacuo* and then crystallized from a methanol-ether mixture. Saponification of the ester yielded 94 mg. of crystalline biotin.

Isolation of Radiobiotin from L. arabinosus—To 250 mg. of dried *L. arabinosus* cells were added 150 mg. of non-isotopic *d*-biotin and the mixture was autoclaved in a sealed vessel with 12 ml. of 2 N sulfuric acid for 2 hours. Incorporated in the system was a side arm containing NaOH solution to trap liberated carbon dioxide. The amount of radioactivity in the carbon dioxide liberated was markedly decreased by the addition of the non-isotopic biotin prior to hydrolysis, compared to hydrolysis without added biotin. The hydrolysate was adjusted to pH 5 with NaOH and the biotin was removed by adsorption on charcoal and was purified by the same procedure that was used in the case of the medium. The yield of crystalline biotin was 97 mg.

Radioactivity Analyses—Comparisons of the specific activities of the purified biotin samples from the cells and medium were made with a standard prepared by crystallizing 150 mg. of non-isotopic *d*-biotin in the presence of 3.66 γ of radiobiotin. The samples were counted directly as pads of biotin crystals; these were prepared by dissolving 50 mg. aliquots in 5 ml. of water with a few drops of 4 N KOH, and then acidifying with HCl. The precipitated samples were collected on filter paper disks in the same apparatus used for the preparation of barium carbonate pads. That the samples contained no appreciable amounts of radioactive impurities was indicated by the identical specific activities shown by these pads and pads prepared from the corresponding purified methyl esters.

The biotin pads were counted by means of a mica window Geiger-Müller tube and associated scaling circuit. The standard biotin preparation (43.6 mg.), the biotin from the cells (45.5 mg.), and that from the medium (45.2 mg.) gave 307, 88, and 240 counts per minute above background, respectively. The values given in Table I are those obtained after correction to zero self-absorption. The carbon dioxide liberated during the hydrolysis of the *L. arabinosus* cells was counted as barium carbonate and was found to contain a negligible amount of radioactivity.

Appreciation is expressed to Miss Mary Lloyd and Mrs. Susan Wing for assistance in carrying out the microbiological assays reported in this paper.

SUMMARY

Biotin labeled with C^{14} in the ureido carbon atom has been synthesized from radioactive phosgene and the diamino acid derived from biotin.

The stability of the ureido ring system of biotin during the biotin-controlled fixation of carbon dioxide by *Lactobacillus arabinosus* was studied. Growth of this organism in the presence of the labeled biotin, under conditions requiring the participation of biotin in carbon dioxide fixation, did not result in any detectable replacement of the C^{14} of the radiobiotin with non-radioactive carbon.

It is concluded that the mechanism whereby biotin promotes carbon dioxide fixation does not involve a transfer of the ureido carbonyl group of the biotin molecule.

BIBLIOGRAPHY

1. Lardy, H. A., Potter, R. L., and Elvehjem, C. A., *J. Biol. Chem.*, **169**, 451 (1947).
2. Shive, W., and Rogers, L. L., *J. Biol. Chem.*, **169**, 453 (1947).
3. Lichstein, H. C., and Umbreit, W. W., *J. Biol. Chem.*, **170**, 329 (1947).
4. Ochoa, S., Mehler, A., Blanchard, M. L., Jukes, T. H., Hoffmann, C. E., and Regan, M., *J. Biol. Chem.*, **170**, 413 (1947).
5. Robertson, P., and Lardy, H. A., Abstracts, Division of Biological Chemistry, American Chemical Society, 113th meeting, Chicago, April 19–23 (1948).
6. Burk, D., and Winzler, R. J., *Science*, **97**, 57 (1943).
7. Melville, D. B., Hofmann, K., and du Vigneaud, V., *Science*, **94**, 308 (1941).
8. Melville, D. B., Rachele, J. R., and Keller, E. B., *J. Biol. Chem.*, **169**, 419 (1947).
9. Lardy, H. A., Abstracts, Division of Biological Chemistry, American Chemical Society, 112th meeting, New York, Sept. 15–19 (1947).
10. Stokes, J. L., Larsen, A., and Gunness, M., *J. Biol. Chem.*, **167**, 613 (1947).
11. Hofmann, K., Melville, D. B., and du Vigneaud, V., *J. Biol. Chem.*, **141**, 207 (1941).
12. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136**, 509 (1940).
13. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, **139**, 675 (1941).
14. Genghof, D. S., Partridge, C. W. H., and Carpenter, F. H., *Arch. Biochem.*, **17**, 413 (1948).
15. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
16. Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, **62**, 175 (1940).

THE LECITHIN, CEPHALIN, AND SPHINGOMYELIN CONTENTS OF PLASMA AND LIVER OF THE FOWL; THEIR METABOLIC INTERRELATIONS AS SHOWN BY THE ADMINISTRATION OF DIETHYLSTILBESTROL

By R. E. RANNEY, C. ENTENMAN, AND I. L. CHAIKOFF

*(From the Division of Physiology, University of California
Medical School, Berkeley)*

In 1944, Taurog *et al.* (1) pointed out that practically all of the phospholipides in plasma of man and dog contain choline. This indicates that the plasma phospholipides of these two species consist almost entirely of lecithins and sphingomyelins. Confirmation of this finding has now come from Hack (2) and from Sinclair (3–5). The former reported that about 95 per cent of the phospholipides in the serum of normal human subjects are choline-containing and Sinclair found that 3 to 8 per cent of the plasma phospholipides in man, dog, beef, and pig are cephalins.

The composition of plasma phospholipides of the bird differs, however, from that of the mammal. Thus, Flock and Bollman (6) reported that only 53 per cent of the plasma phospholipides of the domestic fowl are choline-containing, whereas Sinclair (3–5) has shown that in turkey serum no more than 80 per cent of the phospholipides are of this type.

By combining two methods, (1) that developed by Taurog *et al.* (7) for separating choline-containing and non-choline-containing phospholipides by permanent adsorption of the latter upon MgO and (2) that of Schmidt *et al.* (8), based on the selective destruction of lecithins in a solution of choline-containing phospholipides, it became possible to measure separately the concentrations of lecithins, cephalins, and sphingomyelins in tissues. These methods were used in an earlier study dealing with dog tissues (9) and in the present investigation they have been applied to a study of plasma phospholipides of the domestic fowl. In order to investigate further the metabolic significance of the three types of phospholipides found in the bird, the plasma of the domestic fowl injected with stilbestrol was also examined. This procedure has been shown to induce a lipemia not only in the non-laying female bird but also in the male bird (10).

EXPERIMENTAL

Treatment of Animals—Single comb white Leghorn-New Hampshire cross-bred chickens purchased on the open market were used in this inves-

tigation. A few single comb white Leghorns obtained from the Poultry Division of this university were also used. All birds were immature

TABLE I

Phospholipide Fractions in Plasma of Normal and Diethylstilbestrol-Injected Immature Single Comb White Leghorn-New Hampshire Crossbred Male Fowl

	Bird	Weight	Age	Total phos-pholip-ides	Lecithins	Sphin-gomy-elins	Choline-containing phospholipides		Cephalins
							Per cent of total phospholipides		Per cent of total phospho-lipides
							Lecithins	Sphin-gomy-elins	
		gm.	*wks.*	*mg per cent*	*mg per cent*	*mg. per cent*			*mg per cent*
Control	A	740	14	196	131	22	66.7	11.2	43	21.5
	D	920	14	167	112	21	67.6	12.6	34	21.0
	E	1100	15	204	140	31	68.5	15 2	33	16.0
	H	1010	15	128	75	26	58.5	20.3	27	21.1
	N	920	9	152	98	14	64.2	8.8	41	27.0
	R	1360	12	124	80	18	64.2	14.8	26	21.0
Mean				162	108	22	64.9	13.8	34	21.3
s.e.*							3.59	3.96		3.40
Injected†	K	940	9	1000	705	60	70.5	6.0	235	23.5
	L	920	9	1130	855	15	75.6	1.3	260	23.1
	M	840	9	1020	750	12	73.5	1.2	258	25.3
	O	920	9	865	652	25	75.4	2.8	188	21.8
	P	1500	12	387	309	20	79.8	5.2	58	15.0
	Q	1420	12	805	575	63	71.5	7.7	167	20.8
	S	1390	12	1030	695	121	67.5	11.7	214	20.8
	T	1440	12	1030	720	51	69.9	5.0	259	25.1
	U	1360	12	1140	778	72	68.2	6.4	290	25.4
Mean				934	671	54	72.4	5.1	214	22.3
s.e.*							3.98	3.32		3.09

' Standard error $= \sqrt{\dfrac{1}{(n-1)} \sum_{i=1}^{n} (x_i - \bar{x})^2}$.

† The injected birds received a total of 5 mg. per kilo of stilbestrol in corn oil intramuscularly. This amount was administered in three equal doses over a 24 hour period. The control birds received simultaneously equivalent amounts of corn oil alone.

males, ranging in age from 9 to 15 weeks at the time they were sacrificed.

Diethylstilbestrol was injected intramuscularly. The crystalline material was dissolved in corn oil and administered in a concentration of 2

mg. per cc. The amounts injected are recorded in Tables I and II. The control birds received only corn oil. The total dose recorded was divided into three equal portions: the first was injected between 8.00 and 9.00 a.m.; the second, between 4.00 and 5.00 p.m. of the same day; and the third, between 8.00 and 9.00 a.m. of the next day.

Blood was removed by heart puncture 48 hours after the first injection

TABLE II

Phospholipide Fractions in Livers of Normal and Diethylstilbestrol-Injected Immature Single Comb White Leghorn-New Hampshire Crossbred Male Fowl

	Bird*	Liver weight	Total phospholipides, per cent fresh tissue	Choline-containing phospholipides				Cephalins	
				Per cent fresh tissue		Per cent of total phospholipides		Per cent fresh tissue	Per cent of total phospholipides
				Lecithins	Sphingo-myelins	Lecithins	Sphingo-myelins		
		gm.							
Normal	E	36.69	2.53	1.28	0.09	50.5	3.8	1.16	45.6
	H	29.40	3.11	1.46	0.15	47.0	4.6	1.50	48.4
	N	16.29	3.10	1.41	0.25	45.5	8.1	1.44	46.4
	R	30.76	2.95	1.43	0.18	48.5	6.0	1.34	45.5
Injected†	L	29.39	3.38	1.65	0.22	48.7	6.5	1.51	44.7
	M	22.43	3.52	1.66	0.22	47.2	6.3	1.64	46.6
	O	24.68	3.43	1.80	0.08	52.5	2.3	1.55	45.2
	P	52.25	3.02	1.54	0.07	51.0	2.3	1.41	46.7
	Q	52.79	2.65	1.23	0.17	46.4	6.5	1.25	47.1
	S	44.82	3.12	1.48	0.19	47.5	6.1	1.45	46.4
	T	45.47	3.30	1.74	0.04	52.7	1.3	1.52	46.0
	U	44.93	3.18	1.54	0.20	48.4	6.4	1.44	45.2

* See Table I for body weights and ages.
† See note to Table I.

was made. The birds were then sacrificed by an injection of nembutal, and the livers were removed for analysis.

Immediately after each bird was sacrificed, its liver was removed and thoroughly ground. Samples of the minced tissue (approximately 10 gm.) were then weighed and placed in a flask containing 100 cc. of alcohol. These samples were stored at −18° until analyzed.

Methods

Extraction of Lipides—Plasma was treated for 1 hour, at 55–60°, with 10 volumes of a 3:1 alcohol-ether mixture. The supernatant was filtered and the residue reextracted, for 1 hour, with 5 volumes of the same solvent mixture. The second extract was again filtered and the two extracts

combined. The combined extracts were then concentrated to a small volume at reduced pressure in an atmosphere of CO_2. The concentrate was treated with several portions of petroleum ether and the combined petroleum ether extracts were made to suitable volume with the same solvent.

The lipides were extracted from the liver according to the procedure outlined by Taurog et al. (7).

Separation of Lecithins, Cephalins, and Sphingomyelins—The total phospholipide content of each extract was measured by its phosphorus content. Phosphorus was determined by King's method (11). Choline-containing and non-choline-containing phospholipides were separated according to the method of Taurog et al. (7). In this procedure the phospholipides contained in a petroleum ether extract are adsorbed on MgO. The choline-containing phospholipides are then eluted from the MgO by methanol, while the cephalin fraction remains permanently adsorbed.

The methanol solution of lecithins and sphingomyelins was brought to near dryness, suspended in 1 N KOH, and incubated at 37° for 24 hours according to the procedure of Schmidt et al. (8). This hydrolyzed the lecithins and rendered the phosphorus component of this fraction soluble in 10 per cent trichloroacetic acid. Sphingomyelins are unaffected by this mild hydrolysis.

The amount of cephalin P contained in a petroleum ether extract was calculated as the difference between the total P content of the extract and the P present in the methanol eluate (the fraction having the choline-containing phospholipides). The value for lecithin P is given by the acid-soluble P found after hydrolysis of the methanol eluate. Sphingomyelin P is represented by the difference between the total P of the methanol eluate and the acid-soluble P found after the mild hydrolysis of this eluate.

The reliability of the above procedure for separating sphingomyelin P from lecithin P of plasma has been discussed by Zilversmit et al. (9). Since the value for sphingomyelin P is obtained as a difference between choline-containing phospholipide P and lecithin P, this value is subject to the errors of the individual determinations. The values for these individual determinations were much higher in the stilbestrol-injected birds than in the normal, and therefore the sphingomyelin values reported below for the injected birds are probably more reliable than those of normal birds.

Results

Normal Bird—The composition of plasma phospholipides of the normal male fowl is shown in Table I. Lecithins and sphingomyelins comprise

about 65 per cent and 14 per cent, respectively, of the phospholipides, while cephalins amount to about 21 per cent. Thus, by the use of another method, we have confirmed the observation of Sinclair (3–5) that approximately one-fifth of the plasma phospholipide of the bird is composed of cephalins.

TABLE III

Cephalin, Lecithin, and Sphingomyelin Contents of Livers of Various Animals

Investigator	Species	Per cent of total phospholipides			
		Choline-containing	Cephalins*	Lecithins	Sphingomyelins
Present report..	Fowl, normal		46.5 ± 1.0*	47.9 ± 1.0*	5.6 ± 1.6*
" " ..	Fowl, stilbestrol-injected		45.8 ± 0.8*	49.3 ± 2.3*	4.7 ± 2.2*
MacLachlan et al. (12)	Mouse	58	42		
Hodge et al. (13)	"		42	56	
Welch (14)......	Rat		39	45	
Williams et al. (15).........	"		26	69	5
Artom (16).....	"	57	43		
Entenman and Chaikoff, unpublished	"	60	40		
Taurog et al. (7).	Dog	58	42		
Williams et al. (15)	"		38	58	4
Kaucher et al. (17)	Beef		37	58	5

* See note to Table I.

The composition of liver phospholipides of the normal fowl is recorded in Table II. The amounts of cephalins and lecithins found were nearly equal, whereas sphingomyelins were present to the extent of about 6 per cent. A comparison of the values observed here for the bird liver with reported values for other species is shown in Table III. Apparently the values for the bird are in the range of those found for other animals.

Bird Injected with Stilbestrol—The effects of stilbestrol on the composition of plasma phospholipides of the bird are shown in Table I. After the administration of stilbestrol, the lecithin and cephalin contents of plasma increased about 6-fold, while the concentration of sphingomyelins

showed relatively little change. Considerable variation was observed in the sphingomyelin values in the stilbestrol-treated birds and, therefore, we can draw no conclusion as to whether plasma sphingomyelins are influenced by this estrogen. There can be no doubt, however, that the principal effect of stilbestrol is upon the monoaminophospholipides of plasma.

The amount and distribution of *liver* phospholipides was unaffected by the estrogenic treatment (Table II). This is in agreement with earlier findings of Lorenz *et al.* (18) who showed that the phospholipide content of the liver of hens remained unchanged even during the extreme lipemia of the laying season.

DISCUSSION

The presence of significant amounts of non-choline-containing phospholipides in the plasma of the bird is fully confirmed by the results of this study. Approximately 20 per cent of the plasma phospholipides of the domestic fowl contains no choline, and hence this fraction must be regarded as composed of cephalins. In view of the almost complete absence of cephalins in the plasma of several mammals examined (dog, man, pig, and beef), it is surprising to note that plasma cephalins of the bird are active metabolically, as judged by their response to the injection of stilbestrol.

The lack of a demonstrable response on the part of plasma sphingomyelins to the estrogen injection stands out in sharp contrast to the more than 6-fold increase exhibited by both of the monoaminophospholipides, the lecithins and cephalins. It is interesting to recall here that, in the dog, plasma sphingomyelins are less active than lecithins. In this animal, according to Zilversmit *et al.* (19), the turnover rate of plasma lecithins is more than 5 times that of the sphingomyelins.

Fishler *et al.* (20) have shown that, in the dog, plasma phospholipides are synthesized by the liver. Although appreciable amounts of cephalins exist in the liver of the dog (see Table III), they are not apparently available for release to plasma, as are the lecithins and sphingomyelins. It seems reasonable to infer, for the bird, that the lecithins and sphingomyelins found in its plasma are also synthesized in the liver. But whether its plasma cephalins likewise have a hepatic origin remains to be determined.

SUMMARY

1. In contrast to mammalian plasma (man, dog, pig, and beef), in which almost all phospholipides are of the choline-containing types, the plasma of the normal, immature male fowl contains about 21 per cent

cephalins. Approximately 65 per cent of plasma phospholipides are lecithins and 14 per cent are sphingomyelins.

2. Not only are cephalins present in the plasma of the fowl but, as judged by the response of plasma to the injection of stilbestrol, they also engage in metabolic activities at a rate proportional to that of plasma lecithins. The sphingomyelins of plasma, on the other hand, are less active than cephalins, as judged by the response of plasma to the estrogen.

3. Liver phospholipides in the normal bird are composed of 48 per cent lecithins, 46 per cent cephalins, and 6 per cent sphingomyelins. Their amounts were not changed by the injection of stilbestrol.

BIBLIOGRAPHY

1. Taurog, A., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **156**, 385 (1944).
2. Hack, M. H., *J. Biol. Chem.*, **169**, 137 (1947).
3. Sinclair, R. G., *Federation Proc.*, **6**, 291 (1947).
4. Sinclair, R. G., *J. Biol. Chem.*, **174**, 343 (1948).
5. Sinclair, R. G., *J. Biol. Chem.*, **174**, 355 (1948).
6. Flock, E. V., and Bollman, J. L., *J. Biol. Chem.*, **144**, 571 (1942).
7. Taurog, A., Entenman, C., Fries, B. A., and Chaikoff, I. L., *J. Biol. Chem.*, **155**, 19 (1944).
8. Schmidt, G., Benotti, J., Hershman, B., and Thannhauser, S. J., *J. Biol. Chem.*, **166**, 505 (1946).
9. Zilversmit, D. B., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **176**, 193 (1948).
10. Zondek, B., and Marx, C., *Nature*, **143**, 378 (1939).
11. King, E. J., *Biochem. J.*, **26**, 292 (1932).
12. MacLachlan, P. L., Hodge, H. C., Bloor, W. R., Welch, E. A., Truax, F. L., and Taylor, J. D., *J. Biol. Chem.*, **143**, 473 (1942).
13. Hodge, H. C., MacLachlan, P. L., Bloor, W. R., Welch, E. A., Kornberg, S. L., and Falkenheim, M., *Proc. Soc. Exp. Biol. and Med.*, **68**, 332 (1948).
14. Welch, E. A., *J. Biol. Chem.*, **161**, 65 (1945).
15. Williams, H. H., Kaucher, M., Richards, A. J., Moyer, E. Z., and Sharpless, G. R., *J. Biol. Chem.*, **160**, 227 (1945).
16. Artom, C., *J. Biol. Chem.*, **157**, 595 (1945).
17. Kaucher, M., Galbraith, H., Button, V., and Williams, H. H., *Arch. Biochem.*, **3**, 203 (1943-44).
18. Lorenz, F. W., Chaikoff, I. L., and Entenman, C., *J. Biol. Chem.*, **123**, 577 (1938).
19. Zilversmit, D. B., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **176**, 209 (1948).
20. Fishler, M. C., Entenman, C., Montgomery, M. L., and Chaikoff, I. L., *J. Biol. Chem.*, **150**, 47 (1943).

DETERMINATION OF FREE AND TOTAL CHOLESTEROL BY DIRECT CHLOROFORM EXTRACTION*

By GEORGE R. KINGSLEY and ROSCOE R. SCHAFFERT

(From the Clinical Biochemistry Laboratory, General Medical and Surgical Hospital, Veterans Administration Center, Los Angeles)

Since the Liebermann-Burchard reaction was adopted by Grigaut (1) for the quantitative determination of serum total cholesterol, many modified procedures of the original method have been made. The various procedures have differed in extraction method and in the reagents used in the color development. The separation of cholesterol from its esters with digitonin has had limited application because of the time and labor required in the involved and delicate technique. The errors which are encountered in the Liebermann-Burchard color reaction of different cholesterol and cholesterol ester methods have been described by Sperry (2).

Because of the different methods of extraction in use, the wide range of accepted normal values for total cholesterol, the difficulty of performing cholesterol ester determinations, and the instability of color produced in the Liebermann-Burchard reaction, an investigation of the possible improvement in the method of determination of cholesterol and its ester was undertaken.

The method reported has been satisfactorily used over the past 2 years for the determination of several hundred serum cholesterols and cholesterol esters.

Method

Reagents—
Sulfuric acid, concentrated (c.p. or A. C. S.).
Acetic anhydride (c.p. or A. C. S.).
Magnesium sulfate, anhydrous. Dry U. S. P. magnesium sulfate at 150–200°. Discard caked or melted crystals. Heat slowly, to avoid melting or caking of crystals.
Acetic anhydride-sulfuric acid reagent. Mix cautiously 4 parts of cold acetic anhydride and 1 part of cold sulfuric acid (0–5°) in a stoppered

Erlenmeyer flask placed in powdered ice. Use immediately after mixing.

Chloroform (c.p. or A. C. S.).

Chloroform-digitonin reagent, 0.1 per cent. Add 0.4 gm. of digitonin to 400 ml. of reagent grade chloroform. Mix 5 minutes in a Waring blendor, cool, and mix again for 5 minutes. If a high speed blendor is not available, shake the mixture in a Kahn shaker for 30 minutes and store at 37° for 1 week before using. Shake the mixture each time before use.

Stock cholesterol standard. Dissolve 100 mg. of ash-free cholesterol (m.p. 149°) in chloroform and dilute to 100 ml. at the temperature of the refrigerator where it is stored.

Working cholesterol standard. Dilute 10 ml. of stock standard to 100 ml. with redistilled chloroform in the same manner as the stock standard and store in the refrigerator.

Standardization—Add 1, 2, 3, 4, 5 ml. of working cholesterol standard to photometer cuvettes and dilute each to 5 ml. with chloroform. Add 2 ml. of ice-cold fresh acetic anhydride-sulfuric acid reagent, mix, and read the color as directed in "Procedure."

Procedure

Total Cholesterol—Add 0.2 ml. of serum to 10 ml. of chloroform in a 15 × 150 mm. hollow glass stopper test-tube. Stopper tightly. (Use chloroform-washed corks if glass-stoppered test-tubes are not available.) In the same manner add 0.2 ml. of distilled water to 10 ml. of chloroform for the blank determination, and also to 2 ml. of working cholesterol standard and 8 ml. of chloroform for standard determinations. Shake for 5 minutes in a Kahn shaker. Add 1.5 gm. of anhydrous magnesium sulfate and stopper. Mix immediately to avoid caking of sulfate and again shake 5 minutes in a Kahn shaker. Add 0.5 gm. of fullers' earth and stopper. Mix vigorously 10 seconds by hand and centrifuge immediately for 10 minutes at 3000 r.p.m. Pipette 5 ml. of the clear supernatant chloroform extract into a photometer cuvette and add 2 ml. of freshly prepared acetic anhydride-sulfuric acid reagent. Mix by twirling; do not invert. After mixing, read the color developed at 4 to 7 minute intervals to obtain the maximum reading if the temperature of the reaction mixture is 25°, at 10 to 15 minute intervals at 20°, or at other time intervals as indicated by the temperature (see Fig. 2). Read in the photometer against a chloroform blank set at 100 per cent transmission with 625 mμ light transmission. Use the maximum reading for calculation.

Cholesterol Ester—Add 0.2 ml. of serum and 0.2 ml. of distilled water

for a blank, separately, to two 10 ml. portions of chloroform-digitonin reagent; stopper tightly. Shake for 5 minutes in a Kahn shaker. Add 1.5 gm. of anhydrous magnesium sulfate and stopper. Mix immediately to avoid caking of the sulfate and mix in a Kahn shaker for 5 minutes. Add 0.2 ml. of acetic anhydride and stopper. Mix vigorously by hand 10 seconds, add 0.5 gm. of fullers' earth, and stopper. Mix 10 seconds by hand, and centrifuge 10 minutes at 3000 R.P.M. Continue as directed in the total cholesterol procedure.

Calculations

General Formula—Concentration in mg. per 100 ml. $= K^1(2 - \log$ per cent $T)$.

Total Cholesterol, Ester and Reagent Blank[2]—Substitute per cent T in the above formula.

Cholesterol Ester Correction—(Cholesterol ester minus blank) − (cholesterol ester minus blank) $\times$ 0.8.[3]

Corrected Total Cholesterol—(Total cholesterol minus blank) − (cholesterol ester correction).

Corrected Cholesterol Ester—(Cholesterol ester minus blank) $\times$ 0.8.

EXPERIMENTAL

Simplification of Method of Cholesterol Extraction

In most cholesterol methods hot alcohol-ether or alcohol-acetone is used to extract cholesterol from serum. These solvents must be removed by evaporation before the cholesterol is redissolved in chloroform or acetic anhydride for color development. This procedure involves possible loss of cholesterol by oxidation from overheating, or retardation of color development by water remaining after incomplete drying of the residue from previous extractions. Preliminary extraction with additional solvents other than chloroform increases the possibility of mechanical loss of cholesterol.

When serum is shaken with chloroform, very little coagulation of protein occurs for several minutes. A fine emulsion is formed which is ideal for extraction of the cholesterol. The use of alcohol, acetone, and heat coagulates the serum proteins. The addition of 0.3 ml. of 10 per cent sulfuric acid, 0.3 ml. of 95 per cent ethyl alcohol, or 3 gm. of anhydrous magnesium sulfate to 0.5 ml. of serum before extraction with chloroform

---

[1] $K = 688$ at 20° for Coleman spectrophotometers, models 6 and 14, with No. 6-304B cuvettes.

[2] Reagent blank $= 97$ to 99 per cent T with Coleman spectrophotometers.

[3] 0.8 = relative color correction for the ester when read against the cholesterol standard.

prevented the extraction of 11, 66, and 86 per cent, respectively, of the cholesterol present. If the serum is heated on a water bath 10 to 20 minutes at 100° before extraction, 83 per cent of the cholesterol is not extractable with chloroform.

Direct chloroform extraction is also advantageous, as this extract can be used directly for color development once it is free of water and serum proteins. The water is easily removed by the addition of anhydrous magnesium sulfate, which also precipitates the serum proteins after the cholesterol has entered the chloroform solvent phase. The use of chloro-

TABLE I

Comparison of Color Intensity Developed by Reagents Specified by Various Cholesterol Methods

Temperature 19°; light transmission 625 mμ; total volume of reaction mixture 7 ml.; cholesterol 0.5 mg.

Method	Concentration, H_2SO_4	Acetic anhydride	Acetic acid	Chloroform	Light absorption	Time of maximum color development
	vol. per cent	*vol. per cent*	*vol per cent*	*vol. per cent*	*per cent*	*min.*
Kingsley-Schaffert	5.7	22.9	0 0	71.4	81.0	10
Myers-Wardell (6)	1.6	16.4	0.0	82.0	71.4	45
Macheboeuf-Delsal cholesterol method (5)	0.7	71.0	0.0	28.3	62.5	100
" " ester method (5)	0.7	49.6	0.0	49.6	65.5	100
Schoenheimer-Sperry (4)	3.3	64.4	32.2	0.0	50.0	85
Bloor (3)	1.4	28.2	0.0	70.4	76.5	15

form for the direct extraction of serum cholesterol avoids the use of other solvents, heat for extraction and drying, and the loss of cholesterol by protein precipitation at the time of extraction. Mechanical loss is also decreased, as direct extraction with chloroform eliminates additional steps in the method.

Determination of Optimum Proportion of Reagents for Development of Maximum Color and Color Stability in Liebermann-Burchard Reaction

The method of Bloor (3) and its modifications for the determination of cholesterol are widely used. In this method chloroform is used as a cholesterol solvent in the final color development. In the Schoenheimer and Sperry (4) method glacial acetic acid is used for the final solution of

cholesterol digitonide. This modification was made to stabilize the color development. However, as shown in Table I, much less color is developed, and the color must be measured at exact temperatures and time intervals. Macheboeuf and Delsal (5) investigated the effect of variation of the proportion of reagents in the Liebermann-Burchard reaction upon speed and intensity of color development and concluded that maximum color with free cholesterol was obtained with 2 ml. of chloroform, 5 ml. of acetic anhydride, and 2 drops of N sulfuric acid in 30 minutes
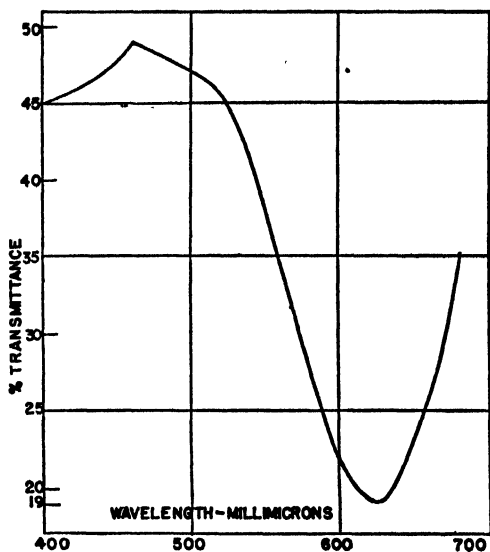
Fig. 1. Spectral transmittance of the color developed by 2 ml. of acetic anhydride sulfuric acid reagent (4:1) added to 0.5 mg. of cholesterol in 5 ml. of chloroform at 0°. Transmittance 80 minutes after color development.

at 25°. The maximum color for cholesterol esters was obtained with 3.5 ml. of chloroform, 3.5 ml. of acetic anhydride, and 2 drops of N sulfuric acid at 25°. Macheboeuf and Delsal also noted that changes in temperature gave different rates of color development as greater color intensity was obtained at higher temperatures.

After testing all possible combinations of reagents, the ratio of reagents as specified in the procedure for the new method was found to give greater maximum color than was yielded by other methods at 19°, as shown in Table I. Light transmission of 625 mμ was used to make these readings, as maximum light absorption for the color developed by the Liebermann-Burchard reaction occurred at this wave-length (see Fig. 1).

Effect of Temperature, Time, Light, Cholesterol Concentration, and Water upon Stability and Intensity of Color Development

The color developed with cholesterol in chloroform solution by the Liebermann-Burchard reaction with the reagents specified in the new method reached approximately the same maximum intensity, regardless of the temperature, as shown in Fig. 2. No correction was made for the change in volume of the reaction mixture at the lower or higher temperatures. At 0° the color reached maximum development at 70 minutes and remained stable for the following 80 minutes at this temperature. At 10° the color was stable at the 30 to 45 minute interval after the start of color development. At 20° maximum color was maintained at the 10 to 15 minute interval after the start of the color development and at 30° at the 3
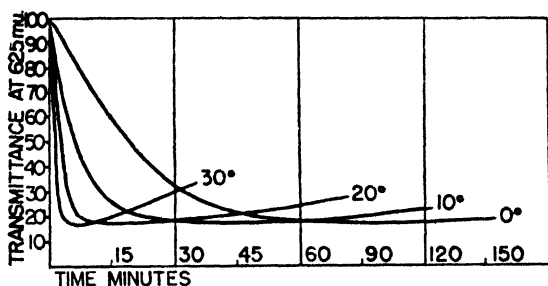
Fig. 2. Effect of temperature on the stability and rate of color development in the Liebermann-Burchard reaction when 2 ml. of acetic anhydride-sulfuric acid reagent (4:1) are added to 0.5 mg. of cholesterol in 5 ml. of chloroform.

to 6 minute interval. The effect of temperature on the intensity, stability, and rate of color development of cholesterol acetate in the Liebermann-Burchard reaction was the same as that on cholesterol, as equal, maximum color density of cholesterol acetate standards was reached in the same time intervals from 0–30°. Maximum color density of chloroform extracts of total cholesterol and cholesterol ester of serum also was reached in the same time intervals at 0–30° as those shown in Fig. 2 for cholesterol. These data indicate that at 20° maximum color development of both cholesterol and its ester will occur between 10 and 15 minutes, which gives ample time for making readings in the spectrophotometer. This is a distinct advantage over other methods, in which color readings must be made at time intervals of 1 minute or less.

The per cent transmission at 625 mμ produced by different concentrations of cholesterol plotted on a semilog scale, as shown in Fig. 3, gave a straight line function from 0 to 500 mg. of cholesterol per 100 ml. of serum. The constant for this curve, including the dilution factors for 0.2

ml. of serum and 5 ml. of the 10 ml. of chloroform solvent used, as outlined in the method was 692 at 0° and 688 at 20°, for substitution in the formula, cholesterol concentration per 100 ml. of serum $= K(2 - \log$ per cent T).

All determinations were carried out in the absence of direct sunlight. Indirect light had no effect on the color development.

Water could be tolerated up to the addition of 0.02 ml. to the reaction mixture with less than 4 per cent loss in color development. Small
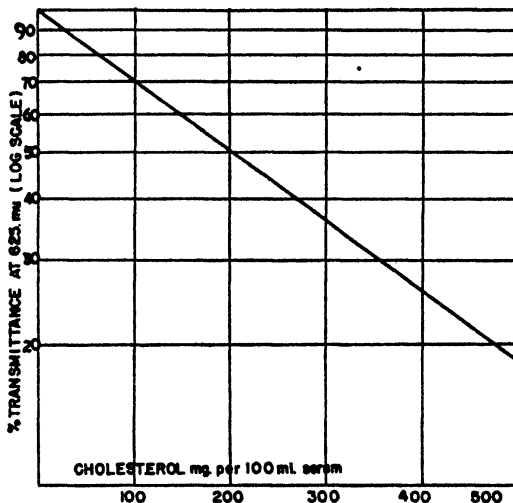
FIG. 3. Relation between cholesterol concentration and per cent transmittance. The curve represents cholesterol standardization in mg. equivalence per 100 ml. of serum for 5 ml. of the 10 ml. chloroform extract of 0.2 ml. of serum. 2 ml. of acetic anhydride-sulfuric acid reagent (4:1) added to 5 ml. of chloroform containing 0.06 to 0.5 mg. of cholesterol at 0°. Transmittance 80 minutes after color development.

amounts of water had no effect on the time of maximum color development.

Simplification of Cholesterol Ester Determination

The determination of cholesterol esters with digitonin by the older methods is a long tedious procedure, which must be carried out with meticulous care. The cholesterol ester determination was incorporated in the simplified direct chloroform extraction to obtain a short, simple accurate method for cholesterol fractionation. This possibility became apparent when it was found that sufficient digitonin[4] was soluble or in

[4] Digitonin preparations from The British Drug Houses, Ltd., Merck and Company, Inc., S. B. Penick and Company, Hoffmann-La Roche, Inc., were found to be equally satisfactory.

suspension in 10 ml. of chloroform to precipitate up to 0.5 mg. of added cholesterol. If the cholesterol concentration is greater than 250 mg. per cent, less serum should be used. A chloroform-digitonin reagent was prepared which was used to extract the cholesterol ester and simultaneously precipitate the free cholesterol directly from the serum as it was extracted. Anhydrous magnesium sulfate and acetic anhydride were added and the cholesterol-digitonin filtered off with the proteins. No overnight standing is required for the complete precipitation or absorption of the cholesterol digitonide. Only cholesterol ester remains in the filtrate, which is determined in the same manner as the total cholesterol. The addition of 0.2 ml. of acetic anhydride to the chloroform-digitonin extract of 0.2 ml. of serum, dried over anhydrous magnesium sulfate, was found necessary for the complete precipitation or absorption of the cholesterol digitonide with the protein precipitate. The addition of less than 0.1 ml. or more than 0.3 ml. of acetic anhydride did not give satisfactory separation of the digitonide. The addition of 0.2 ml. of acetic anhydrid₂ to the dry chloroform extract of 0.2 ml. of serum had no effect on the total cholesterol determination.

Determination of Relative Color Density Developed in Liebermann-Burchard Reaction by Cholesterol and Cholesterol Ester

The ratio of color density developed by the naturally occurring serum cholesterol esters to that of cholesterol was determined by comparing the total cholesterol values obtained on samples of hydrolyzed and unhydrolyzed serum. 0.2 ml. of serum was incubated at 40° with 0.2 ml. of 30 per cent KOH for 30 minutes to convert all cholesterol esters to cholesterol. Hydrolysis was complete at 30 minutes, as a series of hydrolyses at 0, 10, 20, 30, and 60 minutes indicated 98 per cent hydrolysis at 10 minutes and no increase from 20 to 60 minutes. Serum hydrolyzed for 0 minute gave values identical to those from serum to which no hydroxide had been added. The hydrolyzed serum was neutralized (phenolphthalein) with acetic anhydride, 10 ml. of chloroform were added, and the remainder of the procedure carried out as directed in the total cholesterol method. The unhydrolyzed serum developed greater color than the hydrolyzed, the difference indicating the proportionately greater amount of color produced by the cholesterol esters. In twenty-one hydrolyzed and unhydrolyzed serums, presented in Table II, the ratio of color produced by hydrolyzed to unhydrolyzed specimens is quite constant, ranging from 82 to 88 per cent (average 84 per cent). The range of 79 to 68 per cent cholesterol ester of the serums used could cause an additional difference of 2 per cent to the expected 5 per cent experimental error in the ratio of unhydrolyzed to hydrolyzed serum cholesterol. In order to make a correction for the greater color produced by cholesterol ester when

cholesterol standards are used with unhydrolyzed serums, the difference between unhydrolyzed and hydrolyzed serums is subtracted from the color value obtained with serum from which cholesterol has been precipitated with digitonin to obtain a corrected cholesterol ester. A cor-

TABLE II

Ratio of Color Developed by Cholesterol and Cholesterol Ester in Serum of Fasting Normal Subjects and Hospital Patients

Sample No.	Total cholesterol		Cholesterol ester			
	Un-hydrolyzed*	Hydrolyzed*	Uncorrected*	Corrected	Ratio of corrected to uncorrected	In total cholesterol
	mg. per 100 ml.	*mg. per 100 ml.*	*mg. per 100 ml.*	*mg. per 100 ml.*		*per cent*
1	307	270	251	214	0.85	79.0
2	129	106	98	75	0.77	71.0
3	375	321	286	232	0.81	72.0
4	307	268	233	194	0.83	72.4
5	365	316	278	229	0.82	72.1
6	316	263	247	194	0.79	73.7
7	164	137	125	98	0.79	71.5
8	365	312	294	241	0.82	77.4
9	181	149	140	108	0.77	72.4
10	303	254	240	191	0.80	75.1
11	184	154	148	118	0.80	76.5
12	170	140	135	105	0.78	75.0
13	164	137	128	101	0.79	73.6
14	178	149	140	111	0.79	74.1
15	224	193	174	143	0.82	74.1
16	214	175	168	129	0.77	73.6
17	230	193	183	145	0.79	75.0
18	355	290	294	229	0.78	78.7
19	298	244	240	186	0.78	76.4
20	114	95	84	65	0.77	68.4
21	224	190	166	132	0.80	69.4
Average					0.796	

* Average of duplicate determinations.

rection averaging 0.8 is obtained with twenty-one serums (Table II) from a ratio of the corrected to the uncorrected cholesterol ester. As shown above, this correction would also be approximately the same at all temperatures from 0–30° if optimum time at a specific temperature is allowed for maximum color development.

Other laboratories should determine their own correction factor for cholesterol ester, as their reagents, conditions, and technique of analyses may vary from those of the authors.

The correct cholesterol ester is calculated as follows: cholesterol ester (as read against the cholesterol standard) — (the unhydrolyzed total cholesterol minus the hydrolyzed total cholesterol) = the corrected cholesterol ester.

TABLE III

Recovery of Cholesterol and Cholesterol Acetate Added to 0.2 Ml. Samples of Pooled Serums

Sample No.	Original	Added	Recovered	Recovery
		Cholesterol in serum		
	mg.	*mg.*	*mg.*	*per cent*
1	0.060	0.04	0.040	100.0
2	0.077	0.08	0.078	97.5
3	0.163	0.12	0.124	103.3
4	0.117	0.16	0.152	95.0
5	0.163	0.16	0.156	97.5
6	0.149	0.20	0.196	98.0
7	0.111	0.24	0.228	95.0
8	0.163	0.28	0.288	102.7
9	0.133	0.32	0.312	97.5
10	0.111	0.36	0.344	95.5
11	0.163	0.36	0.364	101.0
12	0.102	0.48	0.474	98.5
13	0.102	0.61	0.600	98.3
14	0.102	1.00	1.008	100.8
15	0.113	1.00	1.040	104.0
16	0.102	2.00	2.046	102.3
17	0.102	3.00	3.126	104.2
Average ...				99.5
		Cholesterol ester in serum		
18	0.340	0.252	0.246	97.8
19	0.340	0.504	0.486	96.2
20	0.340	0.630	0.602	95.5
21	0.340	1.260	0.121	96.0
22	0.340	2.520	2.530	100.0
23	0.340	3.780	3.860	102.2
Average .				97.9

Determination of Accuracy of Method by Recovery Experiments

0.04 to 3.8 mg. of cholesterol and cholesterol acetate was added to 0.2 ml. samples of fresh pooled serum and the per cent recoveries determined by comparison with the cholesterol and cholesterol ester originally present in the serum. The recoveries, shown in Table III, are all within 5 per cent of the expected 100 per cent recovery.

TABLE IV

*Partition of Serum Cholesterol in Fasting Normal Adults**

The results are given in mg. per 100 ml.

Sample No.	Sex	Total cholesterol			Ester			Per cent
		Specimen I	Specimen II	Average	Specimen I	Specimen II	Average	
1	M.	257	265	261	201	204	203	77.8
2	"	172	172	172	135	131	133	77.5
3	"	218	218	218	165	160	163	74.7
4	"	221	215	218	169	169	169	77.5
5	"	177	183	180	132	136	134	74.4
6	"	206	212	209	156	156	156	74.6
7	"	160	165	163	118	122	120	73.7
8	"	161	165	163	119	121	120	73.7
9	"	250	250	250	189	195	192	76.8
10	"	173	179	176	128	128	128	72.8
11	"	215	209	212	164	160	162	76.5
12	"	240			170			70.8
13	"	185			136			73.5
14	"	195			140			71.8
15	"	213			155			72.7
16	"	222			172			77.5
17	"	248			178			71.8
18	"	231			168			72.6
19	"	197			141			71.5
20	"	240			168			70.0
21	"	251			190			75.7
22	"	182			143			78.5
23	"	246			191			77.5
24	"	218			166			76.0
25	"	236			178			75.4
26	"	192			143			74.5
27	"	187			149			80.2
28	"	217			168			77.4
29	"	226			179			79.3
30	"	232			186			80.1
31	"	233			186			79.9
32	F.	212	212	212	156	156	156	73.6
33	"	189	189	189	136	141	139	73.5
34	"	158	164	161	116	116	116	72.0
35	"	177	179	178	133	133	133	74.8
36	"	202	196	199	158	153	156	78.3
37	"	172	178	175	133	133	133	76.0
38	"	207	207	207	162	156	159	76.7
39	"	156	161	159	116	118	117	73.6
40	"	242	242	242	196	196	196	81.0
41	"	262	249	256	186	182	184	72.0
42	"	205	211	208	166	171	169	81.0

TABLE IV—*Concluded*

Sample No.	Sex	Total cholesterol			Ester			Per cent
		Specimen I	Specimen II	Average	Specimen I	Specimen II	Average	
43	F.	228	220	224	168	168	168	75.0
44	"	202	202	202	146	146	146	72.4
45	"	162	157	160	129	133	131	81.8
46	"	173	176	175	138	146	142	81.1
47	"	195	195	195	158	158	158	81.0
48	"	183	178	181	137	133	135	74.6
49	"	245			181			73.8
50	"	244			178			73.0

* Determinations on 20 to 50 year-old individuals 12 hours after evening meal.

TABLE V

Comparison of Direct Chloroform Extraction Method with Schoenheimer-Sperry Method for Determination of Total Cholesterol and Esters on Serum of Hospital Patients

Sample No.	Direct chloroform extraction						Schoenheimer-Sperry (4)		
	Cholesterol-digitonin			Water-digitonin			Cholesterol		
	Cholesterol			Cholesterol					
	Total	Ester	Ester	Total	Ester	Ester	Total	Ester	Ester
	mg. per 100 ml.	mg. per 100 ml.	per cent	mg. per 100 ml.	mg. per 100 ml.	per cent	mg. per 100 ml.	mg. per 100 ml.	per cent
1	226	150	66	225	155	69	238	164	69
2	188	136	72	186	146	78	192	136	71
3	170	107	63	178	99	56	167	105	63
4	213	143	68	219	153	70	216	159	73
5	183	130	71	187	138	74	184	132	72
6	245	173	71	244	178	73	245	178	73
7	156	91	58	157	90	57	157	88	56
8	109	70	64	105	67	64	105	67	64
9	306	228	74	307	221	72	302	220	73
10	188	136	72	188	136	72	187	134	72
11	161	119	74	160	123	77	158	117	75
12	231	165	71	230	170	74	230	166	72
13	116	63	54				119	61	51
14	164	84	51				159	88	55

Determination of Normal Range of Serum Cholesterol and Cholesterol Esters

Determinations of serum total cholesterol and cholesterol ester were carried out in duplicate on 50 fasting normal adults (Table IV). Single and average duplicate determinations for both sexes were within the range of 159 to 261 mg. of total cholesterol, and 116 to 203 mg. of cholesterol ester per 100 ml. of serum. The per cent cholesterol ester ranged in narrow limits, from 70.0 to 81.8 per cent. Duplicate determinations of

cholesterol and cholesterol ester agreed to within 5.0 per cent or less. These values are in close agreement with those of Sobel and Mayer (7), who found normal serum total cholesterol to range between 160 and 267 and cholesterol ester between 118 and 202 mg. per 100 ml. of serum. The per cent ester levels were within the narrow limits found by Schoenheimer and Sperry (4) and more recently by Clarke and Marney (8) and Sobel and Mayer (7).

Comparison of New Method with Method of Schoenheimer and Sperry (4) for Determination of Cholesterol and Cholesterol Ester

The procedure, as directed under "Method," was compared with that of Schoenheimer and Sperry. An additional comparison for ester determination was made by use of a saturated solution of digitonin in water (0.5 to 0.8 per cent), with a slight modification of the procedure described for the chloroform-digitonin reagent, which is as follows: 0.2 ml. of serum and 10 ml. of chloroform are shaken 5 minutes and 0.2 ml. of digitonin in water (5 per cent) added. The mixture is shaken again for 5 minutes. 1.5 gm. of anhydrous magnesium sulfate is added and the mixture shaken for 5 minutes. 0.3 ml. acetic anhydride is added, shaken 5 minutes, and the remainder of the determination continued as directed in the cholesterol ester procedure with the chloroform-digitonin reagent. A comparison of these two methods with that of Schoenheimer and Sperry is shown in Table V, in which the determinations were made upon serum of fasting hospital patients.

SUMMARY

1. A simple, rapid, accurate method for the determination of serum cholesterol and cholesterol esters by direct extraction with chloroform has been presented, in which evaporation, drying, and ester hydrolysis are not required and a cholesterol fractionation is completed within 1 to 2 hours. It has been assumed that only cholesterol and its esters in chloroform extracts of serum are responsible for the development of color in the Liebermann-Burchard reaction.

2. The optimum proportion of reagents in the Liebermann-Burchard reaction has been determined in order to obtain maximum and equal rate of color development for both cholesterol and cholesterol esters in the same interval of time.

3. Approximately the same maximum color intensity is obtained by both cholesterol and cholesterol ester at different temperatures, as the temperature affects only the rate of the reaction. The time for the measurement of maximum color is determined by the temperature of the reaction.

4. The accuracy of the method has been confirmed by recovery experi-

ments, by close agreement of duplicate determinations, and by comparison with the method of Schoenheimer and Sperry.

5. Total serum cholesterol in 50 normal adults ranged from 159 to 260 mg. per 100 ml. and cholesterol ester from 70 to 82 per cent as determined by the new method.

BIBLIOGRAPHY

1. Grigaut, A., *Compt. rend. Soc. biol.*, **118**, 827 (1910).
2. Sperry, W. M., and Brand, F. C., *J. Biol. Chem.*, **150**, 315 (1943).
3. Bloor, W. R., *J. Biol. Chem.*, **24**, 227 (1916).
4. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1939).
5. Macheboeuf, M., and Delsal, J. L., *Bull. Soc. chim. biol.*, **24**, 10 (1942).
6. Myers, V. C., and Wardell, E. L., *J. Biol. Chem.*, **36**, 147 (1918).
7. Sobel, A. E., and Mayer, A. M., *J. Biol. Chem.*, **157**, 255 (1945).
8. Clarke, D. H., and Marney, A. F., *J. Lab. and Clin. Med.*, **30**, 615 (1945).

ISOTOPICALLY LABELED NUCLEIC ACID FROM YEAST

By FREDERICK J. Di CARLO and ALFRED S. SCHULTZ

(From The Fleischmann Laboratories, Standard Brands, Inc., New York)

and PAUL M. ROLL* and GEORGE BOSWORTH BROWN*

(From the Laboratories of the Sloan-Kettering Institute for Cancer Research, New York)

The preparation of isotopically labeled nucleic acid was undertaken to provide material for a study of nucleic acid metabolism and a source of labeled nucleotides and nucleosides, which are otherwise unavailable. For this purpose yeast was propagated in a manner found to produce a rich source of nucleic acid (1) on a medium supplying excess N^{15} in the form of ammonium sulfate. Nucleic acid was isolated by adapting the method of Redfern (2) to laboratory usage. From the nucleic acid were isolated adenine picrate, guanine sulfate, and silver pyrimidines. The isolated purines and pyrimidines contained essentially identical amounts of isotopic nitrogen (Table I). This level was slightly lower than the N^{15} content of the nucleic acid sample and was considered indicative of contamination of the nucleic acid sample by some proteinaceous material. The residual yeast (from which nucleic acid was extracted) showed a higher N^{15} content than did the nucleic acid. This could be attributed to the fact that there was proportionately more protein produced during the growth period, since the seed yeast contained 7.22 per cent nucleic acid (dry basis), whereas the final yeast contained only 6.37 per cent nucleic acid (dry basis).

The molasses employed for the yeast propagation contained 20.93 gm. of nitrogen. It was calculated from the isotope analyses that 3.99 gm. (19.0 per cent) of this nitrogen was assimilated by the yeast.

EXPERIMENTAL

Yeast Propagation—420 gm. of moist yeast (*Saccharomyces cerevisiae*) (29.08 per cent solids, 8.91 per cent N (dry basis), and 7.22 per cent nucleic acid (dry basis)) were used as seed. This yeast was suspended in 21 liters of water to which 21.0 gm. of H_3PO_4 and 210 gm. of $(NH_4)_2SO_4$ (5.79 atom per cent excess N^{15}) were added. Molasses (2100 gm.) was added by the continuous process described by Hayduck (3). The wort was aerated

vigorously during the 8 hour propagation period, and the pH was maintained within the range of 4.8 to 5.5 by adding NaHCO₃ as required. The yeast was separated from the spent wort and washed by centrifugation. It weighed 1680 gm. and contained 28.01 per cent solids, 9.37 per cent N (dry basis), and 6.37 per cent nucleic acid (dry basis).

Nucleic Acid Isolation—1675 gm. of the washed yeast were suspended in 3.8 liters of cold 2.3 per cent NaOH solution and stirred mechanically for 1 hour at 10°. Concentrated HCl (135 cc.) and 45 gm. of Filter-Cel were added. The pH was brought to 6.6 with glacial acetic acid, and the mixture was centrifuged at 2500 R.P.M. for 15 minutes. After decanting the supernatant liquor, the residue was washed with 1 liter of water.

Calcium chloride (300 gm.) was dissolved in the combined solutions. The mixture was made acidic to Congo red by the addition of HCl. The precipitate of crude calcium nucleate was filtered and washed with alcohol.

TABLE I

Isotopic Content of Isolated Products

	Atom per cent excess N¹⁵
Yeast nucleic acid .	3.43
Protein residue . .	3.84
Purine hydrochlorides	3.36
Adenine. .	3.36
Guanine.........	3.34
Silver pyrimidines........	3.32

The filter cake was extracted by stirring for 45 minutes at 70° with 430 cc. of water containing 36 gm. of sodium acetate. The hot mixture was filtered and the residue washed with water. The combined filtrate and washings were diluted with 200 cc. of alcohol, and the pH was brought to 1.1. After stirring for 30 minutes to permit complete precipitation of nucleic acid, the product was collected by filtration, washed with alcohol, and dried *in vacuo* over NaOH. The yield was 21.5 gm.

Isolations of Purines and Pyrimidines—1 gm. of the nucleic acid was hydrolyzed in methanol by hydrogen chloride gas according to the method of Levene (4), yielding 0.180 gm. of purine hydrochlorides. From these the adenine and guanine were separated as described by Levene, and 0.063 gm. of once recrystallized guanine sulfate and 0.033 gm. of three times recrystallized adenine picrate were obtained. The methanolic solution from which the purine hydrochlorides had precipitated was used for the preparation of mixed silver pyrimidines according to the procedure reported by Barnes and Schoenheimer (5), except that NaOH was used instead of

Ba(OH)$_2$ for neutralization. After the brown precipitate of silver pyrimidines and silver oxide was thoroughly washed, it was dried with ethanol and ether and without further treatment was digested for N^{15} determinations.

SUMMARY

It was demonstrated that yeast may be employed as a convenient source of isotopically labeled compounds of biochemical interest. In this work yeast was propagated on a medium containing excess N^{15} in the form of ammonium sulfate. Isotopically labeled nucleic acid was isolated, and it was shown that the isotopic nitrogen was uniformly distributed throughout the purines and pyrimidines.

BIBLIOGRAPHY

1. Di Carlo, F. J., and Schultz, A. S., *Arch. Biochem.*, **17**, 293 (1948).
2. Redfern, S., U. S. patent 2,387,040 (1945).
3. Hayduck, F., U. S. patents 1,449,103 and 1,449,105 (1923).
4. Levene, P. A., *J. Biol. Chem.*, **53**, 441 (1922).
5. Barnes, F. W., Jr., and Schoenheimer, R., *J. Biol. Chem.*, **151**, 123 (1943).

THE METABOLISM OF YEAST NUCLEIC ACID IN THE RAT

By PAUL M. ROLL* AND GEORGE BOSWORTH BROWN*

(From the Laboratories of the Sloan-Kettering Institute for Cancer Research, New York)

AND FREDERICK J. DI CARLO AND ALFRED S. SCHULTZ

(From The Fleischmann Laboratories, Standard Brands, Inc., New York)

Recent studies on the fate of labeled dietary purines in the rat have shown that dietary adenine is a precursor of the adenine and of the guanine of tissue nucleic acids (1), but that guanine (2), hypoxanthine (3), and xanthine (3) are not utilized for nucleic acid synthesis. Similar experiments have shown that the pyrimidines, uracil, thymine (2), and cytosine (4) are not incorporated into the nucleic acids of the rat. However, orotic acid (4-carboxyuracil), while not a nucleic acid component, is utilized as a precursor of the uracil and cytosine of nucleic acids (5). Since none of the pyrimidines occurring in nucleic acids are utilized when they are present in the diet, it seems probable that derivatives of these compounds are first synthesized in a way which does not involve the participation of these free pyrimidines, but which may involve orotic acid (6), and that these derivatives are then incorporated into the nucleic acids. Such derivatives could be the pyrimidine nucleosides or nucleotides. Because free guanine is not a precursor of nucleic acid guanine, it has been suggested (1) that similar derivatives of the purines may be involved in the conversion of adenine to nucleic acid guanine. Up to now, no nucleic acid component more complex than the purines or pyrimidines has been studied by the tracer technique. The availability of ribonucleic acid labeled with isotopic nitrogen (7) now enables us to extend these studies to nucleic acids.

The fate of exogenous nucleic acids, nucleotides, and nucleosides has been studied in the past by the method of feeding or injecting these compounds into animals in nitrogen balance and determining what changes occur in the quantities of the various constituents of the excreta. In this way it has been found in several species that, following the administration of nucleic acids, the nitrogen of the purines could be largely, but not always completely, accounted for in the extra allantoin, uric acid, or other free purines (8–11), but that pyrimidines were absent from the urine and that their nitrogen probably had been converted to urea (12, 13). The metabolism

of the nucleotides and the nucleosides has been studied in the same way. The nitrogen of purine nucleosides has been found to be extensively excreted as uric acid or allantoin (14, 15), although Cerecedo and Allen found that dogs apparently excrete about one-third of the nitrogen of guanosine as urea (16). It has been shown that the nitrogen of the pyrimidine nucleotides and nucleosides is almost completely excreted as urea, with the appearance of no nucleotides or nucleosides and only small amounts of free pyrimidines in the urine (13, 17). While such experiments are of value in demonstrating the end-products of nucleic acid metabolism, they have yielded no information concerning the pathways of tissue nucleic acid synthesis. This present work is the first of a series of investigations designed to gain further information about nucleic acid metabolism in the rat by the use of labeled nucleic acid and some of its hydrolytic products.

In Experiment I a sample of isotopic yeast (pentose) nucleic acid was fed to two male rats over a period of 3 days. In Experiment II a solution of nucleotides, produced by the alkaline hydrolysis of a sample of the isotopic nucleic acid, was administered to two male rats by intraperitoneal injections which were given three times a day over a 3 day period. In each experiment the compounds were furnished at a level of about 0.4 mM of nucleic acid per kilo of body weight per day. During the experiments the urine was collected and was used for the isolation of allantoin, urea, and ammonia. After the administration of the isotopic compounds was completed, 1 day was allowed to elapse before the animals were sacrificed in order that the compounds might be more completely utilized. Nucleic acids were prepared from the viscera and from them guanine, adenine, and mixed pyrimidines were isolated. A sample of nucleic acid-free muscle protein was prepared. The per cent excess N^{15} in the isolated compounds was determined and these values are shown in Table I.

When the nucleic acid was fed, it was extensively absorbed and metabolized, as indicated by the fact that about 27 per cent of the allantoin excreted during the experiment was formed from the isotopic compound and that appreciable degradation to urea and ammonia occurred. There was a small but definite incorporation of the dietary material into the nucleic acids of the viscera. Because the per cent excess N^{15} in the dietary nucleic acids is about 60 times greater than that in the nucleic acids from the viscera, it would require only about 1 to 2 per cent contamination by the former to account for all of the isotope found in the isolated nucleic acids. However, contamination of such a magnitude does not seem probable, because the time that elapsed between the last feeding and the sacrifice of the animals was sufficient to allow essentially complete digestion and absorption of the dietary material and because the intestines were opened and thoroughly washed before they were added to the other organs.

Although the purines and pyrimidines of the dietary yeast nucleic acid contained equal concentrations of isotopic nitrogen, only 1.0 per cent of both the adenine and the guanine of the nucleic acids from the viscera was derived from the dietary material, while 2.2 per cent of the pyrimidines was derived from it. It seems likely, therefore, that the isotope found in both the purines and pyrimidines of the isolated nucleic acids represents a true incorporation of the dietary material and not a contamination by dietary nucleic acid retained in the gut.

TABLE I

Administration of Nucleic Acid

	Nucleic acid feeding, Experiment I		Nucleotide injection, Experiment II	
	Atom per cent excess N^{15}	Atom per cent excess, calculated on basis of 100 per cent in administered compound	Atom per cent excess N^{15}	Atom per cent excess, calculated on basis of 100 per cent in administered compound
Nucleic acid (dietary)..	3.34*	100		
Nucleotides (injected).....................			3.34*	100
Sodium nucleic acids (viscera)............	0.036			
Nucleic acids (viscera)...................	0.057	1.7	0.153	4.6
Purine hydrochlorides....................	0.037			
Adenine.................................	0.031	1.0	0.125	3.7
Guanine	0.032	1.0	0.126	3.7
Silver pyrimidines........	0.074	2.2	0.170	5.0
Ammonia................................	0.033	1.0	0.07	2.0
Urea...........................	0.058	1.7	0.054	1.6
Allantoin..........	0.886	26.6	0.947	28.3
Muscle protein........................ ...	0.005	0.1	0.008	0.2

* The value for the nucleic acid preparation was 3.43; that for the purines and pyrimidines isolated from it was 3.34 ± 0.02.

In Experiment II, in which a solution of nucleotides prepared from isotopic nucleic acid was given by intraperitoneal injection, it was found that this mixture of nucleotides was more efficiently utilized for the synthesis of nucleic acids than was the dietary nucleic acid. Although equivalent quantities were administered in the two experiments, the nucleic acids isolated after injection of the nucleotides contained 2.7 times more isotope than was present in the nucleic acids obtained in Experiment I. Again, both purines contained the same atom per cent excess N^{15}, but in this case nearly 4 times as much of each was derived from the injected nucleotides. In Experiment II, just as in Experiment I, the incorporation of the iso-

topic material into the pyrimidines of the tissue nucleic acids was greater than the incorporation into the purines, although here the difference in uptake was smaller than it was in Experiment I. The allantoin and urea were derived from the injected compounds to about the same extent as in Experiment I. The level of N^{15} in the urinary ammonia was twice as high as it was in Experiment I and was even higher than the N^{15} level in the urea isolated from the same urine. This may have been due, partly, to a breakdown of other isotopic compounds in the urine to ammonia, because the ammonia was not isolated from this sample of urine until 2 months after it had been collected, and, although it had been stored under toluene in the refrigerator, it is possible that a small amount of allantoin, for example, had been degraded to ammonia.

<div align="center">EXPERIMENTAL</div>

Materials and Methods—The labeled nucleic acid was isolated from yeast which had been cultured in a medium containing isotopic ammonium sulfate as the main source of inorganic nitrogen (7), and was found to contain 3.43 atom per cent excess N^{15}. Adenine, guanine, and mixed pyrimidines were isolated from this nucleic acid and were found to be labeled to the same extent (7). For the preparation of a solution of mixed nucleotides a sample of the isotopic nucleic acid was hydrolyzed by the method described by Buell (18). 1.0 gm. of nucleic acid was suspended in 20 cc. of water and was brought into solution at pH 7 by the gradual addition of 3 N NaOH. After 0.3 gm. of solid sodium hydroxide was added, the solution was diluted to 25 cc. and allowed to stand at room temperature for 24 hours. It was then neutralized to pH 7.5 with 1.0 N HCl, diluted to 50 cc., and stored in the refrigerator until used. This solution contained 0.020 gm. or 0.0153 mM of nucleic acid per cc. as a mixture of nucleotides.

Adult, male, Sherman strain rats were used. They were fed 60 gm. of Rockland Purina chow per kilo of body weight per day, and the nucleic acid preparations were administered at the rate of 0.4 mM per kilo per day, assuming for purposes of calculation a "statistical" tetranucleotide (19) of molecular weight 1300. The maintenance and the method of sacrifice of the animals, the preparation of nucleic acids from the viscera, the isolations of guanine, adenine, and mixed silver pyrimidines, the isolation of the urinary constituents, and the preparation of the muscle protein have been described previously (1).

Nucleic Acid Feeding; Experiment I—Two rats weighing 242 and 258 gm. were used. The lighter animal received 126 mg. of the isotopic nucleic acid and 14.5 gm. of food per day for 3 days, while the other one received 134 mg. of the nucleic acid and 15.5 gm. of food. The food was ground into a paste with water, and the nucleic acid was dissolved in water with the aid

of a minimum of 3 N NaOH and was added to the food. This ration was prepared at the start of the experiment and was kept in the refrigerator. Aliquots were removed each day. The animals ate all of the food, but each rat lost about 10 gm. in body weight during the experiment.

Nucleotide Injection, Experiment II—Two rats weighing 310 and 260 gm. were used. They received 8.1 and 6.8 cc. of the nucleotide solution respectively per day for 3 days. The daily quota of solution was given in three doses of approximately equal volumes by intraperitoneal injection. The animals ate well, but the heavier one suffered a loss of weight of 9 gm., while the lighter animal lost 15 gm. during the experiment. Both rats developed diarrhea. This condition has been noticed before in rabbits, dogs, and cats following the injection of pyrimidine nucleotides and sodium nucleic acid (9, 13). At sacrifice the lighter animal was found to be suffering from peritonitis. The intestines from this animal, which were quite inflamed, were not added to the organs utilized for the preparation of nucleic acids.

DISCUSSION

The extent to which the sample of dietary nucleic acid was digested prior to absorption cannot be ascertained, but it is probable that a mixture of nucleotides, nucleosides, and perhaps purines was produced. It would seem, therefore, that in both of the experiments described here nucleotides or nucleosides were the compounds available for cellular metabolism. If the results of these experiments are compared with the results of the experiments in which adenine was fed (1), it can be seen that the adenine in adenylic acid or adenosine is apparently less efficiently utilized by the rat for nucleic acid synthesis than is the free purine. In the earlier experiment adenine was fed to rats at a level of 0.2 mM (27 mg.) per kilo per day, and it was found that 5.4 per cent of the adenine and 3.2 per cent of the guanine in the nucleic acids of the viscera had been formed from the dietary compound and that these values were increased to 13.7 and 8.2 per cent, respectively, when the level of administration was increased to 1.5 mM (200 mg.) per kilo per day. In these experiments the animals received approximately 0.4 mM of adenine per kilo per day in the form of nucleic acid or adenylic acid, but only 1.0 per cent of both the nucleic acid adenine and guanine was derived from the dietary nucleic acid and only 3.8 per cent of each was formed from the injected nucleotides. This less efficient incorporation of adenine into nucleic acids when administered to rats as nucleotide or nucleoside rather than as free purine cannot be due to a failure of these compounds to be metabolized, since the high isotope levels in the urinary allantoins gives evidence that extensive absorption and metabolism of the purine moieties had occurred in each case. It is more likely that

partial enzymatic deamination of the adenylic acid or adenosine had occurred before these compounds could be utilized for nucleic acid synthesis. It has been demonstrated that an adenosine deaminase present in intestinal secretions can cause extensive conversion of adenosine to inosine (20–22). This same enzyme has been detected in a number of other tissues (23); so that the parenterally administered compound could also have been partially deaminated. It is significant that the injected nucleotides were nearly 4 times more effective as purine precursors than was the ingested nucleic acid.

In both of these cases, it was found that, in the nucleic acids which had been isolated from the viscera, the ratio of adenine to guanine which had been derived from the isotopic compounds was 1:1. This is in sharp contrast to the results that have been obtained in earlier experiments. When free adenine was the labeled precursor and the nucleic acids of the combined viscera were investigated, this guanine to adenine ratio was invariably close to 0.6:1.0 (1).[1] The significance of this observation is not immediately apparent, but it may indicate that nucleic acid guanine need not necessarily be formed from adenine but can also be derived from other precursors.

All of the purines and pyrimidines present in ribonucleic acid have been tested in the rat and none have been found to be precursors of nucleic acid pyrimidines. Therefore the finding that the nucleic acid pyrimidines are derived from orally administered nucleic acid and from a mixture of injected nucleotides probably implies that the pyrimidine nucleotides or nucleosides are utilized by the rat for the synthesis of nucleic acids. The biological synthesis of nucleic acids in the absence of preformed pyrimidine nucleotides or nucleosides probably involves a synthesis of these units in a way which does not involve the free pyrimidines at any step. The rôle of orotic acid in such syntheses can only be surmised at this time, but it may be that a carboxyl group in position 4 of the pyrimidines is necessary for the attachment of a ribose molecule at position 3.

In both of the experiments described here the nucleic acid pyrimidines were derived from the isotopic compounds to a greater extent than were the purines. However, the difference in uptake that was found following the injection of the nucleotides was smaller than that which was observed after the feeding of nucleic acid. This difference in uptake of the purines and the pyrimidines is not necessarily indicative of any difference in the rates of replacement of the purines and the pyrimidines in the tissue nucleic acids but is probably due to a greater enzymatic degradation of the purine nucleotides or nucleosides than of the corresponding pyrimidine compounds,

---

[1] Furst, S. S., Roll, P. M., and Brown, G. B., unpublished work.

which allows more of the latter to be available for nucleic acid synthesis. Purine nucleosidase, adenosine deaminase, and guanosine deaminase are known to be present in a number of tissues, but pyrimidine nucleosidase has not been found to be as widely distributed (24). Orally administered nucleic acid would probably be subjected to a greater enzymatic attack than would the injected compounds, and the difference in the extent of uptake of the purines and the pyrimidines was found to be greater following this route of administration.

In Experiment I the level of isotope in the urinary urea is nearly twice as great as in the urinary ammonia, which indicates that all of the urea had not been synthesized from body pool of ammonia but that some of it had been formed by direct metabolism of one of the nucleic acid fragments. This result was not noted in Experiment II, but the uncertainty concerning the reliability of the isotope value for this ammonia makes any interpretation of this difference impossible.

In the case of a mutant of *Neurospora crassa*, Pierce and Loring (25) have observed marked antagonistic effects of certain nucleosides or nucleotides on the utilization of others, notably an inhibition of the utilization of cytosine derivatives by adenosine and the reversal of that inhibition by uridine. In view of such interrelationships the results obtained with the mixture of nucleotides must be interpreted with caution and studies of the individual derivatives are desirable.

SUMMARY

Yeast nucleic acid, in which the purines and pyrimidines were equally labeled with N^{15}, has been fed to rats and has been found to be a precursor of both the purines and the pyrimidines of the nucleic acids isolated from the viscera. A sample of this nucleic acid, after hydrolysis to nucleotides, was administered to rats by intraperitoneal injection and was found to serve to a somewhat greater extent as a precursor of the purines and pyrimidines of the tissue nucleic acids.

A material present in yeast nucleic acid, presumably pyrimidine nucleotides or nucleosides, in contrast to free pyrimidines, may serve as a precursor of the pyrimidines of tissue nucleic acids.

The purines of dietary nucleic acid or of injected mixed nucleotides are less extensively utilized for nucleic acid formation than is the free purine adenine.

BIBLIOGRAPHY

1. Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., *J. Biol. Chem.*, **172**, 469 (1948).
2. Plentl, A. A., and Schoenheimer, R., *J. Biol. Chem.*, **153**, 203 (1944).
3. Getler, H., Roll, P. M., Tinker, J. F., and Brown, G. B., *J. Biol. Chem.*, **178**, 259 (1949).
4. Bendich, A., Getler, H., and Brown, G. B., *J. Biol. Chem.*, **177**, 565 (1949).
5. Bergström, S., Arvidson, H., Hammarsten, E., Eliasson, N. A., Reichard, P., and von Ubisch, H., *J. Biol. Chem.*, **177**, 495 (1949).
6. Mitchell, H. K., Houlahan, M. B., and Nye, J. F., *J. Biol. Chem.*, **172**, 525 (1948).
7. Di Carlo, F. J., Schultz, A. S., Roll, P. M., and Brown, G. B., *J. Biol. Chem.*, **180**, 329 (1949).
8. Loewi, O., *Arch. exp. Path. u. Pharmakol.*, **45**, 157 (1901).
9. Mendel, L. B., Underhill, F. P., and White, B., *Am. J. Physiol.*, **8**, 377 (1903).
10. Mendel, L. B., and Brown, E. W., *J. Am. Med. Assn.*, **49**, 896 (1907).
11. Hunter, A., and Givens, M. H., *J. Biol. Chem.*, **17**, 37 (1914).
12. Mendel, L. B., and Myers, V. C., *Am. J. Physiol.*, **26**, 77 (1910).
13. Wilson, D. W., *J. Biol. Chem.*, **56**, 215 (1923).
14. Thannhauser, S. J., and Bommes, A., *Z. physiol. Chem.*, **91**, 336 (1914).
15. Thannhauser, S. J., and Schaber, H., *Z. physiol. Chem.*, **115**, 170 (1921).
16. Cerecedo, L. R., and Allen, F. W., *J. Biol. Chem.*, **107**, 421 (1934).
17. Emerson, O. H., and Cerecedo, L. R., *J. Biol. Chem.*, **87**, 453 (1930).
18. Buell, M. V., *J. Biol. Chem.*, **150**, 389 (1943).
19. Gulland, J. M., Barker, G. R., and Jordan, D. O., *Ann. Rev. Biochem.*, **14**, 175 (1945).
20. Levene, P. A., and London, E. S., *J. Biol. Chem.*, **83**, 793 (1929).
21. Bielschowsky, F., and Klemperer, F., *Z. physiol. Chem.*, **211**, 69 (1932).
22. Zittle, C. A., *J. Biol. Chem.*, **166**, 499 (1946).
23. Conway, E. J., and Cooke, R., *Biochem. J.*, **33**, 479 (1939).
24. Sumner, J. B., and Somers, G. F., Chemistry and methods of enzymes, New York (1943).
25. Pierce, J. G., and Loring, H. S., *J. Biol. Chem.*, **176**, 1131 (1948).

DISTRIBUTION OF C¹⁴ IN GLYCINE AND SERINE OF LIVER PROTEIN FOLLOWING THE ADMINISTRATION OF LABELED GLYCINE*

By PATRICK D. GOLDSWORTHY, THEODORE WINNICK, AND DAVID M. GREENBERG

(From the Division of Biochemistry, University of California Medical School, Berkeley)

In their study of glycine metabolism in rats, Greenberg and Winnick (1) failed to observe the formation of serine from labeled glycine, this failure being due most probably to the relatively low specific radioactivity of the glycine then available, coupled with the large self-absorption factor entailed in the counting of the serine as the p-hydroxyazobenzene-p-sulfonate derivative. Subsequently this transformation was noted with C¹⁴-labeled glycine in rat liver homogenates (2) and slices (3), in yeast grown in a medium containing C¹³-labeled glycine (4), and in rats fed C¹³-labeled glycine and C¹⁴-labeled formate (5).

In repeating our *in vivo* experiments, with use of carboxyl-labeled and methylene-labeled glycine of relatively high specific activity, and by means of the carrier technique, it was concluded that the transformation of glycine to serine in rat liver is extensive and extremely rapid.

In order to reduce to a minimum any possible error due to traces of contaminants, the amino acids isolated with the aid of carriers were recrystallized repeatedly and then converted into one or more derivatives.

EXPERIMENTAL

1 mg. of $NH_2C^{14}H_2COOH$ (4.57 μc. or 560,000 counts per minute per mg.) was injected intraperitoneally into a 200 gm. male rat, and 1.3 mg. of $NH_2CH_2C^{14}OOH$ (7.2 μc. or 880,000 counts per minute per mg.) into a second rat. The first animal was killed after 2 hours and the second after 21 hours. Approximately 1.5 gm. of protein powder were then prepared from each liver by the method given in a previous paper (2). 100 mg. portions of the proteins were mixed with 400 mg. of glycine or L-cystine. In experiments with serine carrier (in which two derivatives were prepared), 200 mg. of protein and 800 mg. of DL-serine were employed. The mixtures were hydrolyzed, and the amino acid carriers reisolated, as pre-

viously described (2). The carriers were then recrystallized repeatedly from hot water, and converted into suitable derivatives.

Results

Columns 3 and 6 in Table I give the specific radioactivities of the unhydrolyzed liver proteins and of the glycine, serine, and cystine isolated with the aid of 4 parts of carrier. The values given for the amino acid

TABLE I

Distribution of C^{14} in Liver Protein Following Administration of Labeled Glycine

Substance isolated	Carboxyl-labeled glycine			Methylene labeled glycine		
	No of recrystallizations	Specific activity	Per cent of total C^{14} in protein	No of recrystallizations	Specific activity	Per cent of total C^{14} in protein
(1)	(2)	(3)	(4)	(5)	(6)	(7)
		counts per min. per mg.			counts per min per mg.	
Liver protein, unhydrolyzed		59.2	100		51.4	100
Glycine	5	5.9	39.9	2	6.5	50.5
"	6	7.3	48.9			
Acetylglycine* (m.p. 203–204°)	1	6.6	44.2	6	5.4	42.4
"				7	6.0	47.0
Serine	7	5.0	34.0	6	5.8	45.0
"	8	5.4	36.8	7	5.6	43.2
Phenylisocyanate derivative† (m.p. 163–164°)	3	4.7	32.0	3	5.5	42.3
p-Toluenesulfonyl chloride derivative‡ (m.p. 213°)	3	5.2	35.4	3	4.4	43.1
Cystine	7	0.48	3.2	6	0.43	4.6
"	8	0.50	3.4	7	0.43	4.5
Cuprous mercaptide§		0.37	2.5		0.30	3.2

* Herbst, R. M., and Shemin, D., *Org. Syntheses*, **19**, 4 (1939); m.p., 207–208°.
† Fischer, E., and Leuchs, H., *Ber. Chem. Ges.*, **35**, 3787 (1902); m.p. 165–166°.
‡ McChesney, E. W., and Swan, W. K., *J. Am. Chem. Soc.*, **59**, 1116 (1937); m.p., 212–213°.
§ Graff, S., Maculla, E., and Graff, A. M., *J. Biol. Chem.*, **121**, 81 (1937).

derivatives have been corrected to correspond to the activities of the free amino acids. The figures in Columns 4 and 7 were calculated by dividing the specific activity of each amino acid by that of the original protein, then dividing by the weight ratio of protein to carrier employed, and lastly multiplying by 100 (2).

It is concluded that approximately 45 per cent of the total C^{14} in the protein was due to glycine and 43 per cent to serine when $NH_2C^{14}H_2COOH$ was administered. When $NH_2CH_2C^{14}OOH$ is given, 45 and 35 per cent

were due to glycine and serine, respectively. A small percentage of the C^{14} was found in cystine, most probably derived from serine.

Microbiological analysis gave a value of 4.3 per cent glycine in rat liver protein,[1] while the serine content, as determined by periodate oxidation (6), was found to be 4.1 per cent. Accordingly, for the rat given $NH_2CH_2C^{14}OOH$, the C^{14} concentrations in the glycine and serine were in the ratio of 10.0:10.5 (45/4.3, 43/4.1), while for the rat given $NH_2C^{14}H_2COOH$, the corresponding ratio was 10.5:8.5 (45/4.3, 35/4.1). The approximately equal C^{14} concentrations suggest that glycine and serine are in rapid biological equilibrium in the liver.

SUMMARY

Following the administration of either carboxyl- or methylene-labeled glycine to rats, high concentrations of C^{14} were found in the serine, as well as in the glycine, of liver protein. The serine accounted for 35 to 43 per cent, and the glycine 45 per cent, of the total C^{14} in the liver protein.

BIBLIOGRAPHY

1. Greenberg, D. M., and Winnick, T., *J. Biol. Chem.*, **173**, 199 (1948).
2. Winnick, T., Moring-Claesson, I., and Greenberg, D. M., *J. Biol. Chem.*, **175**, 127 (1948).
3. Siekevitz, P., Winnick, T., and Greenberg, D. M., *Federation Proc.*, **8**, 250 (1949).
4. Ehrensvard, G., Sperber, E., Saluste, E., Reio, L., and Stjernholm, R., *J. Biol. Chem.*, **169**, 759 (1947).
5. Sakami, W., *J. Biol. Chem.*, **176**, 995 (1948).
6. Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **139**, 687 (1941).

[1] This analysis was kindly performed by Dr. J. C. Lewis of the Western Regiona Research Laboratory, Albany, California.

METABOLISM OF THE NITROFURANS

I. ULTRAVIOLET ABSORPTION STUDIES OF URINARY END-PRODUCTS AFTER ORAL ADMINISTRATION

By H. E. PAUL, F. L. AUSTIN,[*] M. F. PAUL, AND V. R. ELLS

*(From the Eaton Laboratories, Inc., Norwich, New York)*

The general antibacterial action *in vitro* of a variety of furan derivatives substituted in the 5 position with a nitro group has been reported by Dodd and Stillman (1, 2). That the presence of the nitro group is essential was established by comparison of the activities with those of the corresponding non-nitrated derivatives. All the nitrofurans except those limited by insolubility exhibited a marked antibacterial action with the exception of nitrofuroic acid. The latter compound, in contrast, was but weakly active.

With the *in vitro* activity of these derivatives firmly established, attention was directed to their possible use in the treatment of systemic infections. A report by Dodd (3) gave in detail the treatment of animal infections with one of the compounds, 5-nitro-2-furaldehyde semicarbazone (Furacin).[1] In marked contrast to their behavior *in vitro*, not all of the nitrofurans gave a chemotherapeutic response in animal infections. An evaluation of more than sixty compounds[2,3] has shown that the presence of a semicarbazone or closely allied grouping as substituent in position 2 of the furan ring is almost uniquely favorable to pronounced response in this respect.
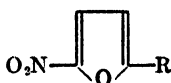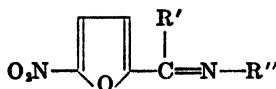
(I)   (II)
Class A   Class B

From a chemical stand-point the nitrofurans prepared to date can be grouped broadly into two general classes. Class A comprises the simpler nitrofurans represented by Formula I where R may be an alkyl, acyl, hydroxyalkyl, or carboxyl group together with the esters or certain other derivatives. Thus Class A would include nitrofuraldehyde and its diace-

---

[*] Present address, Hiram Walker and Sons, Inc., Peoria, Illinois.
[1] Brand of nitrofurazone, "New and nonofficial remedies."
[2] Dodd, M. C., Cramer, D. L., and Ward, W. C., to be published.
[3] Unpublished data.

tate, methyl nitrofuryl ketone, nitrosilvan (5-nitro-2-methylfuran), nitro-furfuryl alcohol and its esters, nitrofuroic acid and its amide and esters, and similar compounds.

Class B (Formula II) comprises those derivatives of nitrofuraldehyde ($R' = H$) or of the nitrofuryl ketones ($R' = $ alkyl or substituted alkyl group) in which condensation has taken place between the carbonyl group and a nitrogen-containing molecule with the elimination of water. Thus in Class B are included the ordinary carbonyl derivatives such as the semicarbazone and oxime as well as more complex analogues which have been prepared in these laboratories.

Although compounds in both Class A and Class B have marked activity *in vitro*, appreciable activity *in vivo* has been exhibited by certain members only of Class B. Chemotherapeutic activity depends not only on anti-bacterial activity of the drug but also on other factors such as its ability to reach the site of infection. Presumably, changes in the compound pro-duced by metabolic processes would explain in part differences between *in vitro* and *in vivo* activity. It was, therefore, of prime importance to submit the nitrofurans to biochemical studies to determine the effect of variations in structure on their metabolism.

As a first step in such a study it was considered desirable to determine the urinary end-products after ingestion of various typical nitrofurans of Class A and Class B. For purposes of comparison it was also considered desirable to feed certain non-nitrated furans.

<div align="center">EXPERIMENTAL</div>

Solvent extraction methods proved of little value in this laboratory in isolating excretory products from the urine after nitrofuran dosage. Chemical methods for the identification and determination of the nitro-furans and their excretory products were not available. Procedures for purifying urine by precipitation were of little help. However, since the nitrofurans have characteristic absorption spectra (4), it was believed that the location and identification of excretory products might be aided by submitting the fresh untreated urine to spectral absorption analysis. This technique proved of great value, and because of its potential useful-ness in related problems of metabolism it is described in some detail here. Although such a method is not suitable for absolute identification of all compounds, it has been extremely useful in indicating the presence or absence of many nitrofurans. In many cases it is reliable for both identi-fication and quantitative analysis of known compounds. After pre-liminary trials the following technique was evolved.

Albino rats of 300 to 400 gm. of body weight were used. The animals were fasted overnight to reduce as far as possible interference from meta-bolic end-products arising directly from foods. Water was supplied *ad*

libitum. Doses of 20 mg. of the drug suspended in simple syrup (sucrose solution) were administered by stomach tube. The animals were caged individually on raised screens over trays covered by waxed paper. Urinary collections by manipulation of the animals were made at 2 hour intervals for a 6 hour period after administration of the drug. Preliminary experiments indicated that appreciable excretion of the drug or its end-products had occurred by this time. If urination occurred before the regular collection, the urine was removed from the waxed paper by medicine dropper. Collections were made in 15 ml. graduated centrifuge tubes and the volume of the urine recorded.

The urines were combined for each drug for the 6 hour period, diluted appropriately, centrifuged, and the optical densities from 450 to 250 mμ (occasionally to 220 mμ) determined, usually at 10 mμ intervals, with the use of a Beckman model D quartz spectrophotometer. Since the dilution required varied with the intensity of the absorbing material excreted and since the volume of urine varied from time to time, it was necessary to correct for volume and dilution. This was done as follows: (1) The optical density at a given wave-length was multiplied by the dilution. This gave the density of the original urine excreted. (2) This value was then multiplied by the volume of the urine excreted. We termed this resultant value "density units." For example, an animal excreted 2.5 ml. of urine in a 6 hour period. The urine was diluted 1:500. The optical density at the desired wave-length was 0.63. Multiplying 0.63 (the optical density) by 500 (the dilution), we obtain 315, the density of the original urine. This value is multiplied by the volume of the urine (315 × 2.5 = 787.5). There were excreted 787.5 "density units" at this chosen wave-length. The total number of "density units" over a period of time is a measure of the total amount of absorbing substance excreted.

By the above method correction values for normal urine have also been determined. Correction for absorption at corresponding wave-lengths due to normal urinary constituents was then made by subtracting the "density unit" values for normal urine from the "density unit" values of the urine of the experimental animals. Urine curves have been plotted with "density units" as ordinates and wave-length in mμ as abscissas.

In the case of identifiable compounds appearing in the urine the method could become quantitative by selecting the wave-length of a major absorption peak and using the following formula,

$$\frac{\text{Density units} \times 10}{E\,_{1\,cm}^{1\%}} = \text{mg. of compound} \qquad (1)$$

when $E\,_{1\,cm}^{1\%}$ is the extinction of a 1 per cent solution of the compound in a 1 cm. cell at the specified wave-length. Solutions of pure furans and

nitrofurans which have been investigated have been found to obey Beer's law up to saturation concentrations.

The average correction curve for a 6 hour period for a representative fasted animal appears in Fig. 1. To show the desirability of fasting the animal before drug dosage, a similar curve for a non-fasted animal is included. In either case absorption becomes great at the lower wave-lengths and care must be exercised in drawing conclusions at wave-lengths at which the correction becomes a large part of the total absorption.

In order to check the reliability of this spectral method, a furan compound whose metabolism had been studied by earlier workers was fed.
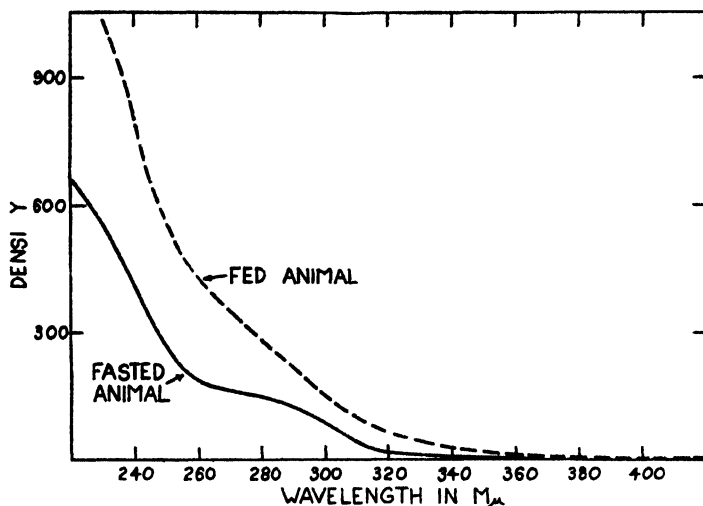
FIG. 1. Ultraviolet absorption characteristics of urine of fasted and fed animals The curves represent the average "density units" per rat for a 6 hour collection period. Solid line, fasted for 15 hours previous to and during urinary collection period. Dashed line, *ad libitum* food consumption before and during urinary collection period.

Schempp (5) fed the sodium salt of furoic acid and was able to account for 70 per cent of the compound as furoylglycine in the urine. In Fig. 2 our results from feeding furoic acid (20 mg. per animal to four rats) are to be seen. The curves for the furoic acid fed and the corrected urine obtained for a 6 hour period are shown. It is apparent that the absorption peak of the material excreted is not that of the furoic acid fed (245 mμ) but that it is at 255 mμ. The spectral curve of a solution of furoylglycine was determined and is included in Fig. 2. The peak for furoylglycine is at 255 mμ and the $E_{1 \text{ cm.}}^{1\%}$ is 864. The shape and peak of the corrected urine curve indicate that the furoic acid ingested is excreted largely as furoyl-

glycine. In order to make the data quantitative, urine collections were made on these rats until the urine curve returned to normal, *i.e.* until little or no furoylglycine was shown spectrophotometrically. The average total "density units" at 255 mμ over this period (10 hours) calculated and corrected for normal urine for the same period were 1693. Therefore, by the use of Formula 1, we obtain (1693 × 10)/864 = 19.6 mg. of furoylglycine. This is equivalent to 13 mg. of furoic acid or 65 per cent of the 20 mg. dose fed, an excellent agreement with the 70 per cent excretion found by Schempp (5).
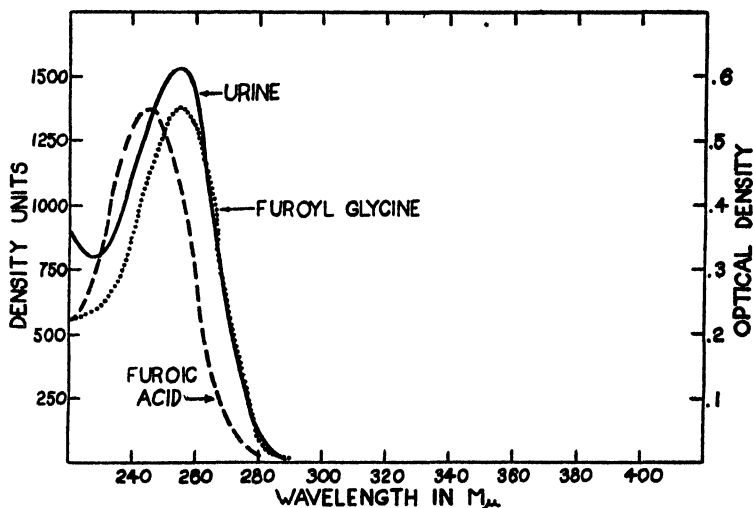

FIG. 2. Ultraviolet absorption characteristics of urine of rat fed 20 mg. of furoic acid compared to absorption curves of furoic acid and furoyl glycine. The urine curve represents average "density units" per rat (after correction for normal urine) for a 6 hour period. Solid line, urine of rat fed furoic acid; dashed line, furoic acid 0.00005 M solution; dotted line, furoylglycine 0.00005 M solution.

Several furan compounds were then studied and spectrophotometric evidence (Table I) has also been obtained to indicate furoylglycine as the major end-product in the urine of rats after feeding furfural, furfural diacetate, furfural hydrazone, furfuryl alcohol, furfuryl propionate, furylacrylic acid, and methylfuryl acrylate. Jaffé and Cohn (6) in 1887 had shown that furoylglycine was an end-product of furaldehyde metabolism. Friedmann (7) identified furoylglycine in rabbit urine after injection of furylacrylic acid and Sasaki (8) found furoylglycine in the urine after feeding furylpropionic acid to dogs. Other end-products were also identified by the above workers after chemical treatment of the urine. It is of

interest to note (Table I) that the urinary end-product after feeding furaldehyde semicarbazone is not furoylglycine.

<div align="center">TABLE I</div>

Ultraviolet Absorption Characteristics of Urine from Rats Fed 20 Mg. Doses of Various Furans

R = [structure] .
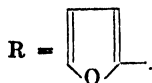
Compound	Absorption maximum	$E_{1\,cm}^{1\%}$	Absorption characteristics of urine	Compound indicated in urine
	$m\mu$			
R—COOH	245	1000	High peak at 255 mμ	Furoylglycine
R—CHO	276	1438	High peak at 255 mμ	"
R—CH(OCOCH$_3$)$_2$	278	393	High peak at 255 mμ	"
R—CH=N—NH$_2$	335	1709	High peak at 255 mμ	"
R—CH$_2$OH	Below 220		High peak at 255 mμ	"
R—CH$_2$OCOC$_2$H$_5$	" 220		High peak at 255 mμ	"
R—CH=CH—COOH	295	1483	High peak at 255 mμ, low shelf in 295 mμ region	" also small amount of furylacrylic acid
R—CH=CH—COOCH$_3$	307	1487	High peak at 255 mμ, low shelf in 290–310 mμ region	Furoylglycine; also small amount of methylfuryl acrylate
R—CONHCH$_2$COOH	255	864	High peak at 255 mμ	Furoylglycine
R—CH=N—NHCONH$_2$	293	1617	High peak at 270 mμ	Not identified

With this background on furan compounds, investigation of the urinary end-products of nitrofuran metabolism was then actively pursued by means of the spectral method for identification or indication of compounds.

Nitrofurans of Class A

The results from feeding various simple nitrofurans of Class A are shown in Table II. The urinary curves obtained from certain typical compounds

appear in Figs. 3 and 4. In Fig. 3 are shown the results from the feeding of nitrofuraldehyde. The shape of the urinary curve and the position of the peak (315 mμ) indicate that this compound is excreted as nitrofuroic acid. The height of the peak (550 "density units") indicates that about 35 per cent of the nitrofuraldehyde fed appears in the urine in 6 hours as nitrofuroic acid.

Since several of the nitrofurans have spectral absorption peaks in the 310 to 320 mμ region, isolation and identification of the material causing the 315 mμ peak in the urine of nitrofuraldehyde-fed rats was attempted. Nitrofuraldehyde was administered in 30 mg. doses to six albino rats, and the urine was collected over a 6 hour period and then treated in a manner similar to that described by Sherwin and Hynes (9) for the isolation of o-nitrobenzoic acid. The urine was adjusted to pH 3.0 to 4.0 with sulfuric acid, reduced to a low volume by lyophilization, and extracted several times with ether. The ether extracts were evaporated to dryness, and the residue taken up in a small amount of hot water and placed in the refrigerator for several days. A few large crystals precipitated and were removed from the mother liquor. Spectral analysis of the mother liquor indicated that it was saturated with nitrofuroic acid.

The crystals were purified by sublimation. The sublimed crystals melted at 185–186°. Pure nitrofuroic acid melts at 185.5°. The melting point of the crystals mixed with pure nitrofuroic acid remained unchanged.

The purified crystals were dissolved in water and the ultraviolet absorption characteristics determined on the Beckman spectrophotometer. The peak was at 315 mμ, the $E_{1\,cm.}^{1\%}$ was 731, and the ratio of the minimum at 260 mμ to the maximum at 315 mμ was 0.191. Pure nitrofuroic acid has a peak at 315 mμ, an $E_{1\,cm.}^{1\%}$ of 725, and a ratio of the minimum at 260 mμ to the maximum at 315 mμ of 0.188. The melting point and absorption data thus confirm the spectral data obtained on the untreated urine and show that nitrofuroic acid is an end-product of nitrofuraldehyde metabolism.

The yield of crystals from this isolation procedure was low. This was thought to be due to poor partition between the water and the ether and to destruction of nitrofuroic acid during the isolation process. However, the existence in urine in addition to nitrofuroic acid of a conjugate with an absorption peak in the same region is not ruled out. The preparation of such a conjugate (i.e. nitrofuroylglycine) has not yet met with success when conventional synthetic procedures are used. This work is not being carried further, since nitrofuroic acid would be the metabolic pathway whether the end-product was nitrofuroic acid or a conjugate.

From the data in Table II, it appears that nitrofuroic · acid, nitrofuraldehyde, nitrofuraldehyde diacetate, nitrofurfuryl alcohol, methyl

TABLE II

Ultraviolet Absorption Characteristics of Urine from Rats Fed 20 Mg. Doses of Various Nitrofurans of Class A

$R = O_2N-$ furan $-$
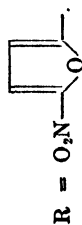
Compound No.	Compound	Absorption maxima (mμ)	$E_{1\,cm}^{1\%}$	Absorption characteristics of urine	Compound indicated in urine	Chemotherapeutic activity†
NF-4	R—COOH	315	726	High peak at 315 mμ	5-Nitro-2-furoic acid	Little or none
NF-2	R—CHO	225, 310	840	" " 315 "	" "	" "
NF-1	R—CH(OCOCH₃)₂	308	465	" " 315 "	" "	Not tested
NF-16	R—CH₂OH	230, 322	715	" " 315 "	" "	" "
NF-15	R—CH₂OCOCH₃	234, 317	563	" " 315–317 mμ	Probably 5-nitro-2-furoic acid	" "
NF-5	R—COOCH₃	304	602	" " 315 mμ	5-Nitro-2-furoic acid	" "
NF-18	R—CH₃	337	875	" " 315 "	" "	" "
NF-41	R—CH₂OCOC₂H₅	232, 317	529	" " 315–317 mμ	Probably 5-nitro-2-furoic acid	Little or none
NF-21	R—COCH₃	308	817	Broad peak at 320 mμ	Not identified; some 5-nitro-2-furoic acid may be present	" "
NF-24	R—COC₂H₅	310	690	" " 320 "	" "	Not tested
NF-13	R—CONH₂	309	880	No peaks, rising absorption below 360 mμ	Not identified; little or no 5-nitro-2-furoic acid	" "
NF-86	R—CN	297	830	No definite peaks, low absorption below 400 mμ	"	" "

NF-112	R—CH=CHCHO	242	355	1012	Slowly rising absorption below 400 mμ, low peak at 280 mμ	Not identified; little or no 5-nitro-2-furoic acid	Not tested
NF-23	R—CH=CHCOOC₂H₅	240	357	645	Broad low band at 310–360 mμ	Not identified; little spectral absorption due to metabolic end-products	Little or none
NF-20	R—H		315	1640	Low absorption below 380 mμ	"	Not tested
NF-33	R—NO₂		307	645	Very little absorption	"	" "

* For longer wave-length.

† *Streptococcus hemolyticus* infections in mice (unpublished data).

‡ Also low peak at 277 mμ.
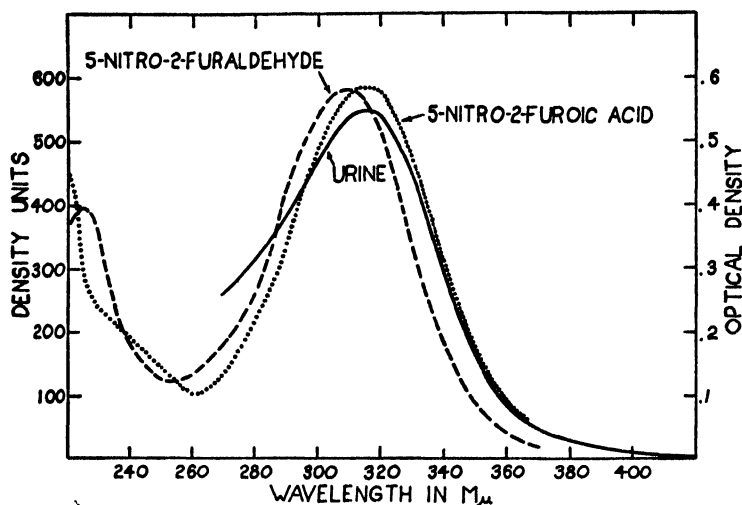
FIG. 3. Ultraviolet absorption characteristics of urine of rat fed 20 mg. of 5-nitro-2-furaldehyde (NF-2). The urine curve represents average "density units" per rat (after correction for normal urine) for a 6 hour period. Solid line, urine of rat fed 5-nitro-2-furaldehyde; dashed line, 5-nitro-2-furaldehyde, 0.00005 M solution; dotted line, 5-nitro-2-furoic acid, 0.00005 M solution.
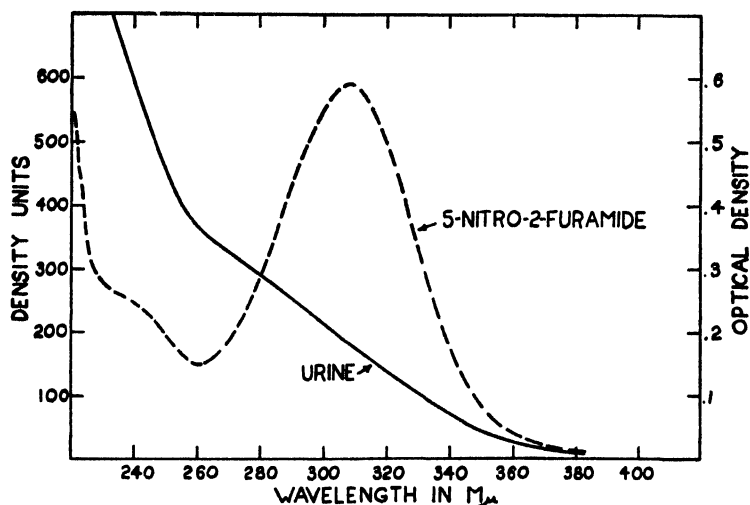

FIG. 4. Ultraviolet absorption characteristics of urine of rat fed 20 mg. of 5-nitro-2-furamide (NF-13). The urine curve represents average "density units" per rat (after correction for normal urine) for a 6 hour period. Solid line, urine of rat fed 5-nitro-2-furamide; dashed line, 5-nitro-2-furamide, 0.00005 M solution.

nitrofuroate, and methyl nitrofuran are all excreted as nitrofuroic acid. Since the urinary absorption curves after the feeding of these compounds indicate little interference from end-products with spectral absorption other than nitrofuroic acid, the data have been treated quantitatively (Table III) as described earlier for furoylglycine. It is apparent that nitrofuroic acid is a major pathway of metabolism of these nitrofurans.

Nitrofurfuryl acetate and nitrofurfuryl propionate are probably also excreted as nitrofuroic acid, but the spectral curves of the three compounds are too similar to determine whether the urinary compound is nitrofuroic acid or the original nitrofuran. From our spectral data we cannot say whether the methyl and ethyl nitrofuryl ketoneš are excreted as nitrofuroic

TABLE III

Urinary Excretion of Nitrofuroic Acid* in Urine in 6 Hours after 20 Mg. Doses of Various Nitrofurans to Albino Rats

R O_2N-

Compound fed	Molecular weight	"Density units" in urine at 315 mμ	Nitrofuroic acid excreted*	Theoretical nitrofuroic acid	Conversion of compound to nitrofuroic acid
			mg.	*mg.*	*per cent*
R—COOH.....	157	1093	15.1	20	75
R—CHO.	141	550	7.6	22	35
R—CH(OCOCH$_3$)$_2$........... ...	243	400	5.5	12.9	43
R—CH$_2$OH.....	143	950	13.1	22	60
R—COOCH$_3$..............	171	770	10.6	18.4	58
R—CH$_3$	127	800	11.0	24.7	45

* Calculated by formula, ("density units" $\times$ 10)$/E_{1\,cm.}^{1\%}$ = mg. of material. The $E_{1\,cm.}^{1\%}$ for nitrofuroic acid at 315 mμ = 726.

acid, for the urinary peak is at 320 rather than 315 mμ and is much broader than expected for a single compound. The data do not exclude the possibility of nitrofuroic acid as one of the end-products.

As a confirmatory procedure on these compounds of Class A the urine of albino rats fed 10 mg. doses of nitrofuraldehyde diacetate was submitted to bacterial assay by a standard cup plate method with *Staphylococcus aureus* as the test organism. If as much as 1 per cent of the drug were excreted, the urine (volume = 2 ml.) should be antibacterial, since *in vitro* activity for this compound is evident on *Staphylococcus aureus* at a concentration of 50 mg. per liter. The results were negative. This lack of antibacterial activity in the urine of animals receiving nitrofuraldehyde

diacetate strengthens the spectrophotometric evidence which indicates excretion of over 40 per cent of the drug as the bacterially inactive nitrofuroic acid.

There are a few compounds of Class A which appear to be metabolized by processes which do not lead to a predominance of nitrofuroic acid. In Fig. 4 the urine curve after the feeding of 5-nitro-2-furamide is shown. When this type of curve is obtained, we cannot draw conclusions as to the identity of the end-products but we can make the following deductions: (1) the compound fed is not excreted in the urine in appreciable quantities; (2) the spectral characteristics denote the absence of other known nitrofurans (*e.g.* nitrofuroic acid) with marked absorption peaks. Other compounds of Class A which are not metabolized predominantly to nitrofuroic acid are nitrofuronitrile, nitrofurylacrylic acid derivatives, nitrofuran, and dinitrofuran.

The conversion of the simpler nitrofurans to nitrofuroic acid *in vivo* finds an analogy with certain non-nitrated derivatives which have been shown to be excreted as furoylglycine, the simple conjugate of furoic acid. The chemical stability of nitrofuroic acid in contrast to that of a number of other nitrofurans permits its ready elimination from the body in appreciable amounts. By the spectral method (Table III) about 75 per cent of the nitrofuroic acid fed has been found in the urine. The low *in vivo* or chemotherapeutic activity (Table II) of certain compounds which exhibit good *in vitro* antibacterial activity may be readily explained for those compounds which are converted to nitrofuroic acid, since nitrofuroic acid has been shown to be only weakly active. From a metabolic standpoint the elimination of certain nitrofurans as nitrofuroic acid finds a parallel in the elimination of *o*-nitrobenzaldehyde as *o*-nitrobenzoic acid (9, 10).

Nitrofurans of Class B

The results from feeding various nitrofurans of Class B are shown in Table IV. The urinary curves obtained after feeding certain compounds of this class are shown in Figs. 5, 6, and 7. Some of the compounds undergo decomposition resulting in a complex series of end-products and some are partially excreted in the urine in unchanged form.

In Fig. 5 is shown the urine of an animal fed β-5-nitro-2-furaldehyde oxime. The major peak of the pure compound is at 346 mμ, but the peak of the material appearing in the urine is in the 335 mμ range.

It is of interest to note here that the α and β forms of the oxime (Table IV) are metabolized to quite different end-products. The breakdown is extensive in either case. Confirmation of the extensive (complete?) breakdown has been obtained by bacterial assay against *Staphylococcus*
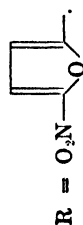
aureus of the urine of albino rats fed these drugs. The urine of rats fed 25 mg. doses of either the α- or β-nitrofuraldehyde oxime exhibited no antibacterial activity, whereas the *in vitro* activity of these drugs is evident at a concentration of 50 mg. per liter (1). These compounds exhibit no *in vivo* activity undoubtedly because they have been changed to inactive forms by metabolic processes. Similarly, it may be seen from Table IV that 5-nitro-2-furaldehyde hydrazone has undergone extensive change and shows no *in vivo* activity.

The chemotherapeutic properties of one of the members of Class B, 5-nitro-2-furaldehyde semicarbazone (Furacin), have been reported by Dodd (3). The urinary curve obtained after feeding this compound is shown in Fig. 6. It is apparent from this curve and from Table IV that extensive breakdown of the drug has occurred during its passage through the animal body. The apparently complete breakdown of this compound was puzzling in view of its *in vivo* activity. However, by refinement of methods and chromatographic treatment of urines it was demonstrated that a portion of the Furacin was in fact being excreted unchanged. This was not obvious from the spectral curve of the untreated urine, since the small amount of compound was masked by the presence of other metabolic end-products with spectral absorption in the same region. With adequate oral doses, unchanged Furacin at an antibacterial level is excreted in the urine. This has been confirmed by isolation of the compound by chromatography. Since other metabolic end-products of the drug isolated to date[3] are without antibacterial activity, the activity of the urine must be due to the presence of the unchanged drug. It is believed that the fact that this nitrofuran escapes complete metabolic transformation within the animal body accounts for its chemotherapeutic activity. The details of the metabolism of Furacin in animals form the subject of a separate report.

A similar picture to that of Furacin (Table IV) is obtained with 5-nitro-2-furyl methyl ketone semicarbazone (NF-57) and with the 5-nitro-2-furyl hydroxymethyl ketone semicarbazone (NF-106). The urine curves indicate complete breakdown of the drug. Nevertheless, in the case of one of these (NF-106), the presence of some of the parent nitrofuran has been demonstrated by further purification of the urine by chromatography.

In Fig. 7 is shown the urinary curve after the feeding of 5-nitro-2-furaldehyde 2-(2-hydroxyethyl) semicarbazone. Enough of this compound appears unchanged in the urine so that its presence is obvious from visual examination of the spectral absorption curve (*i.e.* the similarity of urine peaks to the peaks of the parent compound). Calculation of "density units" would indicate urinary excretion of about 20 per cent of the compound fed. It is thus easy to understand why this compound exhibits

TABLE IV

Ultraviolet Absorption Characteristics of Urine of Rats Fed 20 Mg. Doses of Various Nitrofurans of Class B

R = O_2N—[furan]—.

Compound No.	Compound	Absorption maxima (mμ | mμ)		$E_{1\,cm}^{1\%}$	Absorption characteristics of urine	Compound indicated in urine	Chemotherapeutic activity†
NF-6α	R–CH=NOH	233	340	850	Peak at 435 mμ, urine reddish	Not identified; extensive breakdown	None on α,-β mixture
NF-6β	R–CH=NOH	232	346	850	Peak at 335 mμ	"	None on α,-β mixture
NF-9	R–CH=NNH₂	249	381	825	Low peak at 328 mμ	"	None
NF-7	R–CH=NNHCONH₂	260	375	786	" " or shelves in 270, 320, 400 mμ regions	Several end-products mask presence of small amount of original compound‡	Good
NF-57	R–C(CH₃)=NNHCONH₂	260	375	655	Low broad peaks at 270, 320, 400 mμ	Several end-products; could mask original compound	Fair
NF-106	R–C(CH₂OH)=NNHCONH₂	257	375	540	Broad peak in 370 mμ region, deflection in 260–270 mμ region	Some of original compound§	Good
NF-67	R–CH=NN(CH₂CH₂OH)CONH₂	268	384	670	Peaks at 384 mμ and in 270 mμ region	Original compound present§	"
NF-62	R–CH=NNHCONHCH₃	266	380	734	Peak at 380 mμ, shelf in 260–270 mμ region	Some of original compound present§	Slight

NF-61	R—CH=NN(CH$_2$)CONH$_2$	267 384	760	Peaks in 410 and 285 mμ regions	Other end-products; could mask original compound†	Slight
NF-84	R—CH=NNHCOCONH$_2$	270 362	752	Shelves in 330–380 and 260–270 mμ regions	Some of original compound‡	Good
NF-89	R—CH=NNHCOCONHCH$_2$CH$_2$OH	252,362	704	Shelves in 340–380 and 260–270 mμ regions	Some of original compound‖	"

* For longer wave-length.

† *Streptococcus hemolyticus* infections in mice (Dodd, Cramer, and Ward and other unpublished data).

‡ Chromatography followed by spectrophotometric identification indicated the presence of a small amount of the original compound.

§ Spectral indication confirmed by chromatography and spectrophotometric identification of the original compound in appreciable amount.

‖ Could not be confirmed by chromatography, for column appeared to cause decomposition. See the text.
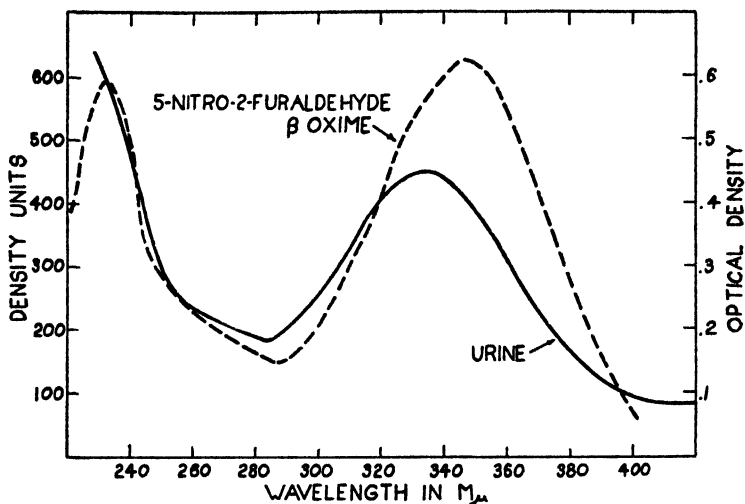
FIG. 5. Ultraviolet absorption characteristics of urine of rat fed 20 mg. of 5-nitro-2-furaldehyde β-oxime (NF-6).   The urine curve represents average "density units" per rat (after correction for normal urine) for a 6 hour period.   Solid line, urine of rat fed 5-nitro-2-furaldehyde β-oxime; dashed line, 5-nitro-2-furaldehyde β-oxime, 0.00005 M solution.
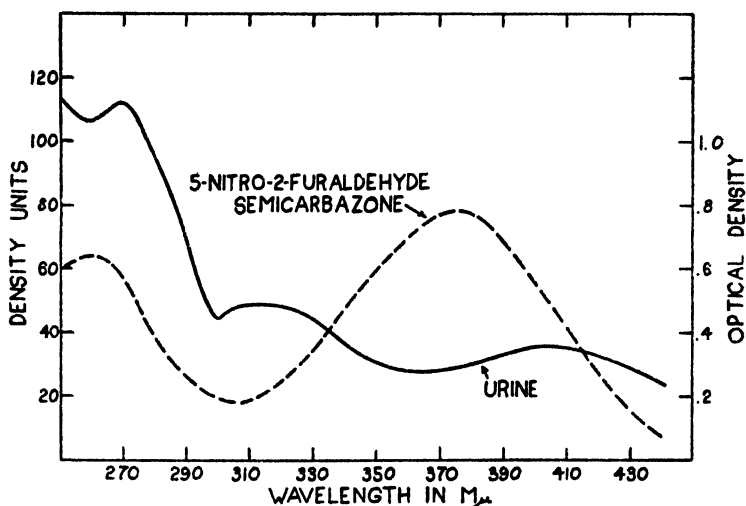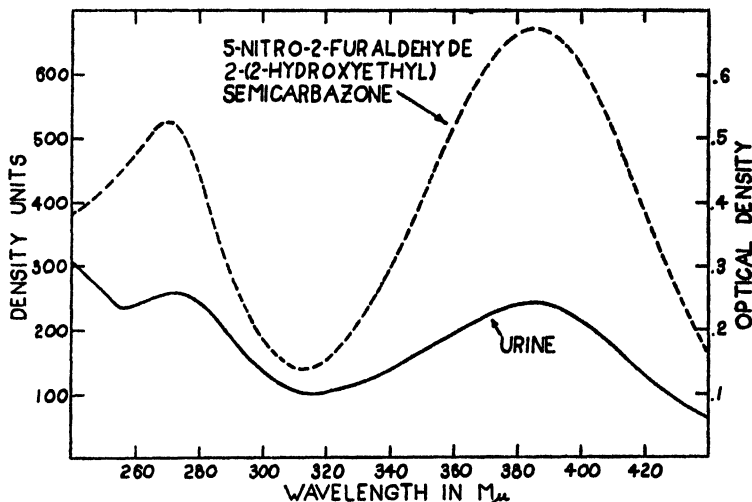

FIG. 6. Ultraviolet absorption characteristics of urine of rat fed 20 mg. of 5-nitro-2-furaldehyde semicarbazone (NF-7).   The urine curve represents average "density units" per rat (after correction for normal urine) for a 6 hour period.   Solid line, urine of rat fed 5-nitro-2-furaldehyde semicarbazone; dashed line, 5-nitro-2-furaldehyde semicarbazone (Furacin), 0.00005 M solution.

good antibacterial activity both *in vitro* and *in vivo*. Inspection of Table IV indicates that 5-nitro-2-furaldehyde 4-methyl semicarbazone (NF-62) is excreted in part unchanged, whereas 5-nitro-2-furaldehyde 2-methyl semicarbazone (NF-61), if excreted, is masked by the presence of other end-products; *i.e.* the peaks in the urine are different from those of the pure compound.

The semioxamazones present a picture similar to that of the semicarbazones. In Table IV it is indicated that these compounds may be excreted in small amounts in the urine, although their presence is partially

FIG. 7. Ultraviolet absorption characteristics of urine of rat fed 20 mg. of 5-nitro-2-furaldehyde 2-(2-hydroxyethyl) semicarbazone (NF-67). The urine curve represents average "density units" per rat (after correction for normal urine) for a 6 hour period. Solid line, urine of rat fed 5-nitro-2-furaldehyde 2-(2-hydroxyethyl) semicarbazone; dashed line, 5-nitro-2-furaldehyde 2-(2-hydroxyethyl) semicarbazone, 0.00005 M solution.

masked by the presence of other end-products. In the case of 5-nitro-2-furaldehyde semioxamazone (NF-84) the presence of a small amount of the parent compound could be demonstrated by chromatography of the urine. This technique was not successful in the case of the 5-nitro-2-furaldehyde 5-(2-hydroxyethyl) semioxamazone (NF-89), for, although a band could first be seen, decomposition appeared to take place as the material went through the column. This nitrofuran is being investigated further.

A more complete study of the effect of various substituents on the stability of the nitrofurans of Class B is under way in these laboratories.

DISCUSSION

The use of spectrophotometric analysis of untreated urine has proved extremely valuable in the study of nitrofuran metabolism. The excretion of appreciable amounts of the compound fed is readily detected by this method (Fig. 7). The excretion of appreciable quantities of breakdown products with ultraviolet absorption characteristics is also readily detected (Figs. 2, 3, 5-7). In some cases a clue to the identity of the end-products is obtained by this method when the urine curves and peaks approximate those of known compounds (Figs. 2, 3). Further refinements of technique are necessary when breakdown products mask the presence of small amounts of compounds (Fig. 6). Other methods must be resorted to for end-products with no ultraviolet absorption characteristics.

From our present results we believe that certain conclusions may be drawn regarding the metabolic pathway of the nitrofurans. The simpler nitrofurans of Class A appear to be metabolized by oxidation to nitro-furoic acid and excreted as such. The Class A compounds such as nitro-furonitrile and nitrofuramide are apparently metabolized in a different manner with extensive breakdown. Further work will be necessary to determine the end-products of such compounds.

The compounds of Class B in some cases are excreted in varying amounts as the compound fed. In other cases they undergo extensive breakdown. In general, some end-products with ultraviolet absorption characteristics are formed. Nitrofuroic acid does not appear to be an end-product of the metabolism of compounds of Class B. The relation of the structure of various nitrofurans to their resistance to breakdown by metabolic processes is under investigation.

SUMMARY

A spectrophotometric method of examination of untreated urines of animals fed various furans and nitrofurans has been described.

By the use of this method furoylglycine has been found as the major urinary end-product after the feeding of furaldehyde, furoic acid, furfural diacetate, furfural hydrazone, furfuryl alcohol, furfuryl propionate, furyl-acrylic acid, and methylfuryl acrylate.

Nitrofuroic acid is believed to be the major urinary end-product after feeding nitrofuraldehyde, nitrofuroic acid, nitrofuraldehyde diacetate, nitro-furfuryl alcohol, nitrofurfuryl acetate, nitrofurfuryl propionate, methyl nitrofuroate, and methyl nitrofuran. The compounds tested which are metabolized to nitrofuroic acid show little or no *in vivo* activity.

The oximes and hydrazone of nitrofuraldehyde are extensively broken down during metabolism. The end-products have not been identified,

but nitrofuroic acid is not indicated as a major metabolite. These compounds have shown little or no chemotherapeutic activity.

Certain semicarbazones and semioxamazones and their substitution products undergo extensive change in the body, while others are excreted in appreciable amounts as the compound fed. Nitrofuroic acid is not indicated as a metabolic end-product of these compounds. In general, these compounds which have shown chemotherapeutic activity have been shown to withstand metabolic breakdown partially and to be excreted in the urine.

The furans and nitrofurans used in this study were synthesized by the Divisions of Chemistry and Chemical Engineering and the chemotherapeutic tests were conducted by the Division of Pharmacology of the Eaton Laboratories, Inc. The authors wish to express their appreciation to John Gillespie and George Everett for their assistance in the numerous spectrophotometric determinations.

BIBLIOGRAPHY

1. Dodd, M. C., and Stillman, W. B., *J. Pharmacol. and Exp. Therap.*, **82,** 11 (1944).
2. Stillman, W. B., and Scott, A. B., U. S. patents 2,416,233-2,416,239 (1947).
3. Dodd, M. C., *J. Pharmacol. and Exp. Therap.*, **86,** 311 (1946).
4. Raffauf, R. F., *J. Am. Chem. Soc.*, **68,** 1765 (1946), and a paper in press.
5. Schempp, E., *Z. physiol. Chem.*, **117,** 46 (1921).
6. Jaffé, M., and Cohn, R., *Ber. chem. Ges.*, **20,** 2311 (1887).
7. Friedmann, E., *Biochem. Z.*, **242,** 288 (1931).
8. Sasaki, I., *Biochem. Z.*, **25,** 272 (1910).
9. Sherwin, C. P., and Hynes, W. A., *J. Biol. Chem.*, **47,** 297 (1921).
10. Quick, A. J., *J. Biol. Chem.*, **96,** 83 (1932).

PREPARATION OF HIGH POTENCY OXYTOCIC MATERIAL BY THE USE OF COUNTER-CURRENT DISTRIBUTION*

BY ARTHUR H. LIVERMORE AND VINCENT DU VIGNEAUD

(From the Department of Biochemistry, Cornell University Medical College, New York City)

The active principles of the posterior lobe of the pituitary gland have been separated and concentrated in several different laboratories by a variety of procedures. In a recent review (1) the details of many of these procedures and the potencies of the products obtained have been compared. Among the methods used for separating and concentrating the oxytocic and pressor factors, differential precipitation has been the most widely used. Electrophoresis of active material has been used effectively in concentrating the pressor factor (2), but this technique has not yet been applied to the purification of the oxytocic substance. The pressor and oxytocic factors have also been separated by chromatographic adsorption (3). Further purification (4) of the material eluted from the chromatographic column has yielded pressor and oxytocic material having a higher potency than that obtained by any of the other methods used up to that time. Although the various concentration procedures have yielded oxytocic and pressor material of quite high potency, there has been no evidence that any of the preparations have been pure.

In this Laboratory the counter-current distribution technique of Craig (5) has recently been applied to the purification of the oxytocic factor. By the use of this technique much inert material has been removed, and there is some evidence that the most potent preparations obtained by this method may be very nearly pure. Starting with material having a potency of 20 units per mg., a product having an activity of 850 units per mg. or higher has been obtained repeatedly. This activity is in the same range as that reported by Potts and Gallagher for the oxytocic material which they prepared (4), which had a potency of 700 units per mg.

Since the oxytocic hormone is soluble in 1-butanol and 2-butanol as well as in water, it is possible to distribute it between one of these alcohols and an aqueous solution. The partition coefficients of the oxytocic material between 2-butanol and various aqueous solutions were determined, and, from the solvent pairs tested, two were selected for use in the purification

procedure described here. These solvent pairs were 0.05 per cent acetic acid-2-butanol, in which the partition coefficient of the active material was found to be 0.4, and 2-butanol-0.01 M ammonium hydroxide, in which the partition coefficient was found to be 1.8.

During the determination of the partition coefficients in various solvents, it was found that, when 2 M phosphate buffer at pH 6.8 was used as the aqueous phase, about 85 per cent of the oxytocic activity but only 50 per cent of the solids went into the 2-butanol layer. Use was made of this fact in effecting a preliminary purification of the starting material. In this way the potency was increased from 20 to around 200 oxytocic units per mg. This partially purified material was then put through several distributions in the Craig counter-current machine (5). The most potent material obtained in this manner had an activity of 865 oxytocic units per mg. when assayed by the chicken blood pressure method (6).

The close agreement of the counter-current distribution curves of weight and activity with the calculated theoretical curve for a single pure substance makes it appear either that the oxytocic material of this high potency is very nearly pure or that, if the material is contaminated, the impurity has a distribution coefficient identical with that of the oxytocic factor.

<center>EXPERIMENTAL</center>

Starting Material—The pitocin preparation which was used in this work possessed 20 units of oxytocic activity per mg. It was obtained in solution[1] containing 5.6 mg. of dry solids per ml. The original activity of this solution in November, 1940, was 200 oxytocic[2] and 4 pressor units per ml. By January, 1947, when the present research was begun, the activity had decreased to 100 units per ml.[2] Assays on this material were made at intervals from January to June, 1948, with the chicken blood pressure method. The average activity was found by this method to be 120 oxytocic units per ml. or about 20 units per mg. of solids.

Partition Coefficients—The partition coefficients (K) of the oxytocic activity were determined with 2-butanol and a number of aqueous solutions. Weighed samples of pitocin (12 to 16 mg.) were equilibrated between the two solvents (3 ml. of each solvent), the layers were separated, and the activity and weight of material in each phase were determined. The results are shown in Table I.

Phosphate buffer (2 M)[3] at pH 6.8 was also tried as the aqueous phase

---

[1] We are indebted to Dr. Oliver Kamm of Parke, Davis and Company, who furnished us with this material.

[2] This assay was carried out in the laboratory of Dr. Oliver Kamm of Parke, Davis and Company by the guinea pig uterine strip method.

[3] Made up from 2 M NaH_2PO_4 (379 ml.) and 2 M K_2HPO_4 (621 ml.).

with both 1-butanol and 2-butanol as the organic phase. In both cases the oxytocic activity was found to be largely insoluble in the buffer. In the case of 1-butanol 100 per cent of the activity and 49 per cent of the total solids were found in the alcohol layer. In the case of 2-butanol 85 per cent of the activity and 47 per cent of the solids were found in the organic phase.

Counter-Current Distribution of Starting Material—212 mg. of dry powdered material obtained by lyophilization of the Parke, Davis and Company "Pitocin" solution were distributed in a 54 tube Craig counter-current machine between 2-butanol and 0.05 per cent acetic acid at 20° with 10 ml. of each solvent per tube. After twelve .transfers had been applied,

TABLE I

Partition Coefficients of Solids and Oxytocic Activity between 2-Butanol and Various Aqueous Solutions

Aqueous phase	pH of aqueous phase	Partition coefficient $(K)^*$	
		Solids	Activity
5% acetic acid. .	3.51	0.6	0.4†
0.05% acetic acid. .	5.24	0.5	0.4†
0.01 M pyridine . .	5.71	0.5	1.4†
0.01 " ammonium hydroxide.. .	7.31	0.5	1.8
0.05 " " "	9.70	0.4	1.4
0.1 " " "	10.01	0.4	1.4
0.1 M triethylamine. . .	9.31	0.4	1.4

* K, ratio amount in upper phase to amount in lower phase.

† Only the material in the butanol phase was assayed. K was calculated from the difference between the total activity of the starting material and the activity found in the butanol phase.

the solution in each tube was evaporated to dryness in a vacuum desiccator at the water pump and was finally dried *in vacuo* over P_2O_5. The resultant glassy product was then weighed, dissolved in 0.05 per cent acetic acid (1 ml. per mg. of solids), and assayed. The results of this distribution are shown in Fig. 1.

A large amount of inert material was found in Tubes 0, 1, and 2, while the active material was found chiefly in Tubes 4 to 8. In this distribution the activity was increased from 20 units per mg. to 188 units per mg. (in Tube 6). The activity recovered from this distribution amounted to 82 per cent of the starting activity.

Preliminary Purification of Starting Material—A preliminary purification procedure for obtaining material with an activity of 200 units per mg. was devised on the basis of our early experience with the distribution of solids

and oxytocic activity between 2-butanol and 0.05 per cent acetic acid and between 2-butanol and 2 M phosphate buffer at pH 6.8.

100 ml. of Parke, Davis and Company "Pitocin" solution were lyophilized to a gummy product. This material was dissolved immediately in 28 ml. of 0.05 per cent acetic acid which had been previously saturated with 2-butanol. This solution was then extracted with six portions of 2-butanol (saturated with 0.05 per cent acetic acid) equal in volume to the acetic acid solution. The butanol extracts were combined and washed three times with equal volumes of 2 M phosphate buffer. The butanol solution
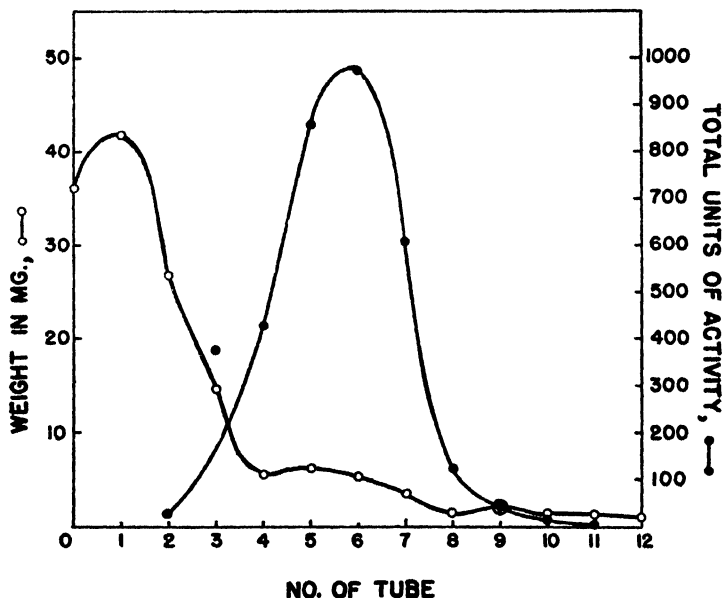
Fig. 1. Counter-current distribution of 212 mg. of material of 20 units per mg. between 2-butanol and 0.05 per cent acetic acid; twelve transfers; O, weight in mg.; ●, total units of activity.

was then dried over dry, powdered magnesium sulfate (1 gm. per 10 ml. of solution) at 5° for 3 to 18 hours. The dry butanol solution was then evaporated *in vacuo* in a water bath at a temperature of not over 25°, and the residue was taken up in 10 ml. of 0.05 per cent acetic acid and lyophilized.

In a typical experiment the lyophilized product was a fluffy white powder which weighed 49 mg. and had an activity of 220 oxytocic units per mg. This is a total of 10,800 units or 90 per cent of the activity present in the starting material. This procedure removed 91 per cent of the solids present in the starting material.

Preparation of Material of 500 Units per Mg. by Counter-Current Distribution of Material of 200 to 250 Units per Mg. (A) Between 2-Butanol and 0.05 Per Cent Acetic Acid—188 mg. of material were prepared from 22.4 gm. of material of 20 units per mg. by the procedure described above. This material had 250 units of oxytocic activity per mg. (total 47,000 units) and was distributed between 2-butanol and 0.05 per cent acetic acid in a 53 transfer distribution at 20°. The solutions from each tube were dried at room temperature *in vacuo*, weighed, and dissolved in 0.05 per cent acetic acid for assay.
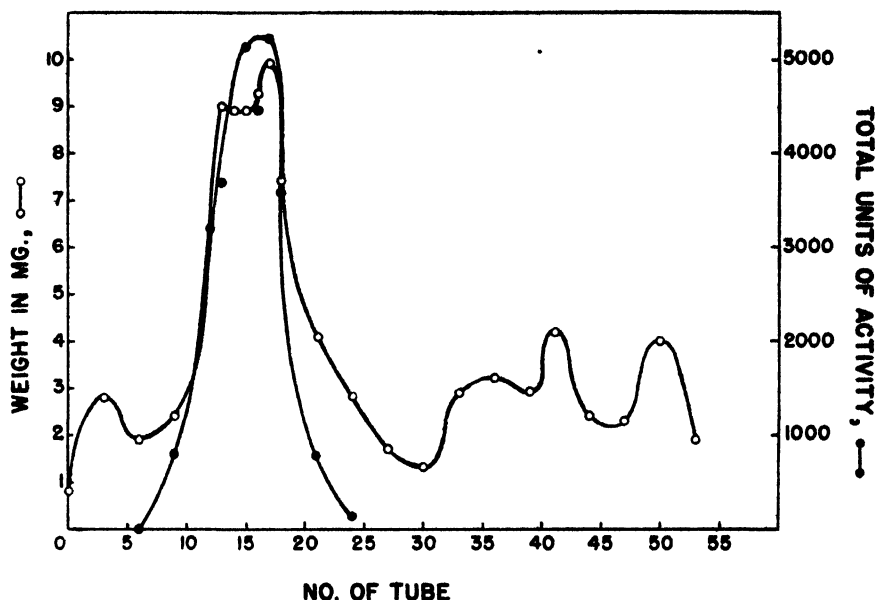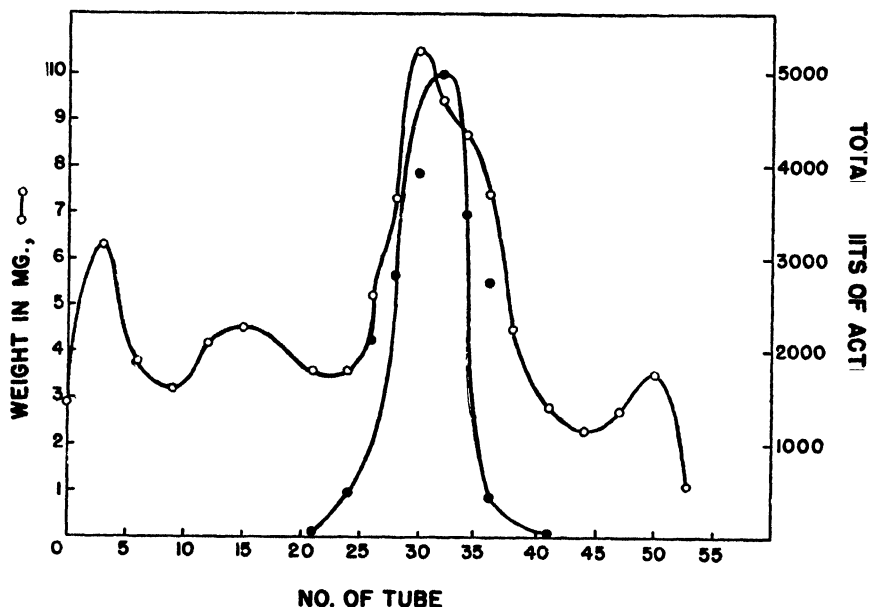

FIG. 2. 53 transfer distribution of material of 250 units per mg. between 2-butanol and 0.05 per cent acetic acid; O, weight in mg.; ●, total units of activity.

The results of this distribution are plotted in Fig. 2. The peak of the activity corresponds with the peak of the major part of the solids. The material in Tube 15 was the most potent, having an activity of 575 units per mg. The total activity recovered from this distribution was 41,000 units or 87 per cent of the starting activity.

(B) *Between 2-Butanol and 0.01 M Ammonium Hydroxide*—234 mg. of oxytocic material having a potency of 205 units per mg. (total 48,000 units) were distributed between 2-butanol and 0.01 M ammonium hydroxide in a 53 transfer distribution at 20°. The solutions were dried and assayed in the usual way. The distribution of solids and activity are shown in Fig. 3. The peak of the activity was in Tube 32. This material had a

potency of 530 oxytocic units per mg.   The total activity recovered from this distribution amounted to 40,600 units or 84 per cent of the starting activity.

Preparation of Material of 700 Units per Mg. by Counter-Current Distribution of Material of 400 Units per Mg.—The material from Tubes 9 through 20 of the 53 transfer distribution between 2-butanol and 0.05 per cent acetic acid was dried to a powder by lyophilization.   The product weighed 80 mg. and contained a total of 38,600 units of oxytocic activity (483 units per mg.).   This material was distributed between 2-butanol and 0.05 per

FIG. 3. 53 transfer distribution of material of 200 units per mg. between 2-butanol and 0.01 M ammonium hydroxide; O, weight in mg.; ●, total units of activity.

cent acetic acid for 100 transfers.   The solutions were dried, weighed, and assayed.   The peaks of both the activity and solid curves were in Tube 30.   The most active material had 700 units of oxytocic activity per mg.

A similar 100 transfer distribution was carried out on the material from Tubes 25 through 38 of the 53 transfer distribution between 2-butanol and 0.01 M ammonium hydroxide.   The combined material from these tubes weighed 105 mg. and contained 39,300 units of oxytocic activity (375 units per mg.).   When this material was distributed between 2-butanol and 0.05 per cent acetic acid for 100 transfers, the peak of the activity and of the solids was in Tube 26.   The material in this tube had a potency of 660 units per mg.

Preparation of Material of 865 Units per Mg. by Counter-Current Distribution—157 mg. of material accumulated from several distributions, and having an oxytocic activity of 335 units per mg. (total 52,500 units), were put through a 53 transfer distribution between 2-butanol and 0.05 per cent acetic acid. Instead of being dried in the usual manner at room temperature, the butanol-acetic acid mixtures were frozen and lyophilized. The resultant white powders were stored *in vacuo* over P_2O_5 until they were dissolved in 0.05 per cent acetic acid and assayed. From this distribution 87 mg. of material having a potency of 550 units per mg. were obtained.

85 mg. of this 550 unit material were distributed again between 2-butanol and 0.05 per cent acetic acid in a 53 transfer distribution. The distribu-
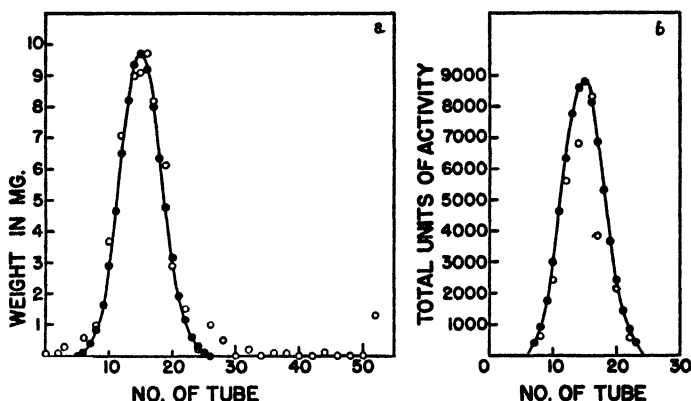

FIG. 4. (a) 53 transfer distribution of material of 550 units per mg. showing experimental values for weights and the theoretical curve for a single substance; O, weight in mg. (experimental); ●, weight in mg. (theoretical). (b) 53 transfer distribution of material of 550 units per mg. showing experimental values for activity and the theoretical curve for a single substance; O, total units of activity (experimental); ●, total units of activity (theoretical).

tion of solids and activity are shown in Fig. 4. The material at the peak of the curve (Tube 16) had a potency of 865 units of oxytocic activity per mg.

Ultraviolet Absorption Spectrum—The ultraviolet absorption spectrum of a solution of purified oxytocic material in 0.05 per cent acetic acid is shown in Fig. 5. Strong end-absorption was found below 240 mμ and there was a small peak at 275 mμ. This peak coincides with the absorption peak of tyrosine and is probably due to tyrosine present in the material (4).

Assay Procedure—The oxytocic material was assayed by a modification of the method of Coon (6). Chickens weighing about 2 kilos were anesthetized with sodium phenobarbital intramuscularly. Injection of oxytocic material was made through a Kaliski needle fastened into one of the large

wing veins. The blood pressure recordings were made with a mercury manometer, the cannula being inserted into a wing artery and connected to the manometer with a rubber tube containing 0.9 per cent sodium chloride. Heparin was used in the saline solution to prevent clotting. A standard solution of oxytocic material was prepared from U. S. P. posterior pituitary reference standard and was made up according to U. S. P. XII directions (7). Injections of this standard solution, which contained 0.5
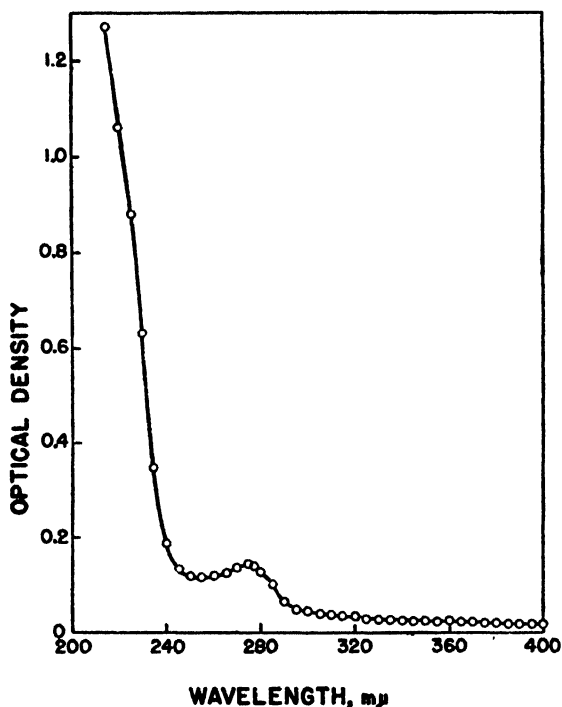

FIG. 5. Ultraviolet absorption spectrum of purified oxytocic material

oxytocic unit per ml., were alternated with injections of unknown oxytocic solutions. The unknown solutions were diluted serially until they contained approximately 0.5 oxytocic unit per ml.

The authors are indebted to Miss Janet Gruschow for assistance with the assays.

SUMMARY

The counter-current distribution principle of Craig has been applied to the purification of the oxytocic fraction of the posterior pituitary gland.

By the use of this procedure, material having a potency of 865 units of oxytocic activity per mg. has been obtained. The characteristics of the distribution curve of this high potency material suggest either that this material is very nearly pure or that, if any impurity is present, it has a distribution coefficient almost identical with that of the oxytocic factor.

BIBLIOGRAPHY

1. Irving, G. W., Jr., and du Vigneaud, V., *Ann. New York Acad. Sc.*, **43**, 273 (1943).
2. Irving, G. W., Jr., Dyer, H. M., and du Vigneaud, V., *J. Am. Chem. Soc.*, **63**, 503 (1941).
3. Potts, A. M., and Gallagher, T. F., *J. Biol. Chem.*, **154**, 349 (1944).
4. Potts, A. M., and Gallagher, T. F., *J. Biol. Chem.*, **143**, 561 (1942).
5. Craig, L. C., *J. Biol. Chem.*, **155**, 519 (1944).
6. Coon, J. M., *Arch. internat. pharmacod.* **62**, 79 (1939).
7. The pharmacopoeia of the United States of America, twelfth revision, Easton, 245 (1942).

α-KETOBUTYRIC ACID AS A PRODUCT IN THE ENZYMATIC CLEAVAGE OF CYSTATHIONINE*

By WILLIAM R. CARROLL,† GARDNER W. STACY, AND VINCENT DU VIGNEAUD

(From the Department of Biochemistry, Cornell University Medical College, New York City)

In the course of investigating cystathionine as a possible intermediate in the metabolic conversion of methionine to cystine, it was shown that both rat liver slices and a saline liver extract are capable of effecting the cleavage of the carbon-sulfur bond of cystathionine with the formation of significant amounts of cysteine (1). It is of course of interest to determine the nature of the other fragment resulting from this cleavage. Two compounds which present themselves as obvious possibilities for this cleavage product are α-aminobutyric acid and homoserine (α-amino-γ-hydroxybutyric acid).

Evidence in support of both of these possibilities is found in some of the recent experimental work in relation to the problem. Binkley (2) prepared a liver extract which was effective in cleaving cystathionine if Mg^{++} or Zn^{++} and adenosine triphosphate were added. He observed that the cleavage of cystathionine with this preparation was accompanied by the disappearance of acid-labile phosphate and the simultaneous appearance of a phosphate compound stable to acid hydrolysis. It was suggested that this product might be phosphohomoserine.

Teas, Horowitz, and Fling, using a mutant strain of *Neurospora* which requires threonine and methionine for growth, found that homoserine could serve in place of both methionine and threonine in supporting the growth of the organism (3). Using other mutant strains, Horowitz (4) had already demonstrated the formation of cystathionine as a step in the synthesis of methionine from cysteine. Teas, Horowitz, and Fling suggested (3) that homoserine might function as the source of the 4-carbon portion of the cystathionine synthesized by the *Neurospora*. Although homoserine may have this relationship to the synthesis of cystathionine in the *Neurospora*, it does not necessarily follow that it is formed in the cleavage of cystathionine by the mammal. It should be borne in mind that there is no evidence that cystathionine can replace methionine in the diet of the rat, even in the presence of choline (5). Furthermore, Armstrong and

---

Binkley (6) have recently demonstrated that DL-homoserine, along with cystine and choline, will not substitute for methionine in the diet of the growing rat.

Some evidence indicating α-aminobutyric acid as a possible cleavage product of cystathionine was furnished by the observations of Dent. By use of paper chromatography he identified α-aminobutyric acid in the urine of a patient suffering from Franconi's syndrome (7). He also noted that α-aminobutyric acid occurred in normal blood and urine (8). The oral administration of methionine to either a normal subject or one with Franconi's syndrome caused an increase in the excretion of α-aminobutyric acid as well as methionine and methionine sulfoxide. Evidence that α-aminobutyric acid may play a rôle in methionine metabolism was recently cited by Fromageot and Clauser (9).

In approaching the question of the nature of the cleavage products of cystathionine, we have encountered the fact that an α-keto acid is formed during the cleavage of this thio ether with a liver enzyme preparation. It is formed along with cysteine and in about equivalent amounts under some conditions. Since the incubation is carried out in the presence of cyanide (1), the formation of keto acid cannot be due to enzymatic decomposition of cysteine to hydrogen sulfide, pyruvic acid, and ammonia (10). An attempt was made to isolate the α-keto acid.

From the combined mixtures from the incubation of several 10 mg. portions of cystathionine with the liver enzyme preparation, we were able to isolate and identify the α-keto acid as α-ketobutyric acid through conversion to its 2,4-dinitrophenylhydrazone. After recrystallization from ethyl acetate the 2,4-dinitrophenylhydrazone melted at 198–199°. It was shown to be identical with an authentic sample of the 2,4-dinitrophenylhydrazone of α-ketobutyric acid by a mixed melting point determination.

The possibility that either homoserine or α-aminobutyric acid could be converted to α-ketobutyric acid under the conditions of the cystathionine cleavage was then investigated. It was shown that indeed homoserine was converted to an α-keto acid by the action of the liver extract under anaerobic conditions; the lactone of homoserine behaved in the same way. The keto acid derived from homoserine was isolated and identified as α-ketobutyric acid through conversion to a 2,4-dinitrophenylhydrazone. This identity was substantiated not only by a mixed melting point determination but also by comparison of its infra-red absorption spectrum with that of an authentic sample. α-Aminobutyric acid did not produce any keto acid upon incubation with the enzyme preparation under the conditions of cystathionine cleavage. Incubation of the enzyme preparation with other compounds related structurally to homoserine, such as threonine and homocysteine, did not result in the formation of any keto acid.

The relationship of the α-ketobutyric acid to the primary cleavage product of cystathionine and the mechanism of this cleavage must rest on future work. If homoserine were formed, the formation of the keto acid would be explainable. On the other hand, the keto acid could well be formed from some other intermediate resulting from the cleavage. Furthermore, the possibility that deamination of the 4-carbon moiety of cystathionine might occur preceding cleavage cannot be ignored.

If, ultimately, it should be demonstrated that homoserine is the primary cleavage product, the excretion of α-aminobutyric acid in the urine in Dent's experiments (7, 8) might be explainable on the basis of the conversion of homoserine to α-ketobutyric acid with subsequent reductive amination of the latter to α-aminobutyric acid. Thus α-aminobutyric acid could result, but that would not necessarily mean that it was a primary cleavage product.

EXPERIMENTAL

Enzyme Preparation and Incubation Procedure

The enzyme preparation used in these experiments was made by a modification of Binkley's method (2) from the livers of adult male albino rats purchased from Rockland Farms. The animals were killed by a blow on the head and bled from the neck. The livers were removed quickly, weighed, placed immediately in 2 volumes of cold 0.9 per cent NaCl in a Waring blendor, and homogenized for 30 to 60 seconds. (Grinding with sand and then mixing with saline proved to be equally as satisfactory as the Waring blendor technique for extracting the enzyme from liver substance.) The mixture was centrifuged for 30 minutes at 3000 R.P.M. in the cold room (5°). The small amount of fat collecting at the top of the centrifuge tubes was removed and the pink supernatant was poured into cellophane tubing for dialysis against 5 to 8 volumes of 0.9 per cent NaCl in the cold with continuous rocking. The pH should not be allowed to drop below 6.5 during the dialysis. Dialysis was continued for 20 to 24 hours, and the saline was changed three or four times during this period. The dialyzed material was then transferred to a flask and heated in a water bath at 55° for 8 to 10 minutes, coagulation of a large amount of protein becoming evident after 5 to 6 minutes. This material was then cooled in an ice-salt bath to 0° and centrifuged in the cold for 30 minutes. The coagulated protein packed firmly, leaving a clear red supernatant which was the enzyme preparation. The pH at this stage was 6.6 to 6.9. In this condition the enzyme is fairly stable when kept in the cold, losing little activity in 3 days and about one-third of the activity in a week. The activity of this enzyme preparation is equal to that of the crude saline extract before dialysis and heat treatment. For the experiments described here, fresh enzyme was prepared every 2 or 3 days to avoid loss of activity. Different preparations

from different groups of three to seven animals showed no appreciable variation in potency.

Destruction of the cysteine formed during cleavage was prevented almost completely by the addition of NaCN, neutralized to a pH of approximately 7, to give a final concentration in the incubation mixture of 0.004 M. The actual concentration of cyanide in the mixture during incubation was less than 0.004 M, since hydrogen cyanide was lost during the equilibration with nitrogen prior to incubation, as described below.

The standard incubation procedure was as follows: The incubation vessels were 50 ml. Erlenmeyer flasks equipped with gas inlet and outlet tubes. In the flasks were placed 1 ml. of the enzyme preparation, 0.2 ml. of 0.1 M NaCN, neutralized to pH 7, the desired amount of substrate, and sufficient 0.067 M phosphate buffer (pH 7.4) to give a final volume of 5.2 ml. At the beginning of each incubation, nitrogen was passed through the flasks for 5 to 10 minutes and the inlet and outlet tubes were then clamped. The flasks were shaken in a constant temperature bath at 38° for 1 hour. This procedure was used for all the incubations, unless other conditions are given. In certain specified cases, the incubations were carried out with distilled water as the medium in place of phosphate buffer. The enzyme effected cleavage under these conditions. The cleavage mixture became slightly cloudy, probably due to the precipitation of some of the protein material.

Because of the low solubility of cystathionine in water it was found convenient, when fairly large amounts of it were used, to dissolve it in a small volume of dilute acid solution, to neutralize this solution with dilute NaOH, and to dilute it with water to obtain a solution of the concentration desired for incubation.

Cystathionine Cleavage Experiments

Various amounts of L-cystathionine were incubated with the enzyme preparation according to the standard procedure, with variations in the amount of enzyme and the incubation time, as indicated in Table I. After incubation, the protein was precipitated by the addition of tungstic acid or trichloroacetic acid. The mixture was then centrifuged and aliquots of the supernatant were used for the estimation of cysteine and keto acid. Cysteine was determined by the Sullivan method (11). The amount of keto acid was determined by application of the direct method for determination of total hydrazones described by Friedemann and Haugen (12). The filtrate from the incubation mixture was treated with 2,4-dinitrophenylhydrazine, alkali was added, and the color development was compared with that from solutions containing known concentrations of the 2,4-dinitrophenylhydrazone of pyruvic acid.

From the summary of the results in Table I, it will be noted that keto acid and cysteine were produced in about equivalent amounts. The extent of cleavage varies with the incubation time and the concentration of enzyme and substrate.

TABLE I

α-Keto Acid and Cysteine Formed in Enzymatic Cleavage of Cystathionine

Cystathionine	Enzyme preparation	Incubation time*	α-Keto acid		Cysteine	
			Amount	Theoretical amount	Amount	Theoretical amount
μM	*ml.*	*hrs.*	*μM*	*per cent*	*μM*	*per cent*
13.5	1	0.33	4.6	35	6.0	44
13.5	1	0.75	6.8	50	7.8	58
13.5	2	0.75	8.0	59	8.0	59
13.5	1	1.0	7.7	57	7.7	57
22.5	1	1.0	9.2	40	10.8	48
45.0	1	1.0	10.9	24	14.6	32
45.0	2	1.0	16.4	36	16.4	36
13.5	1	1.5	8.0	59	7.6	56
13.5	1	2.5	8.0	59	6.4	47

* All incubations were carried out in the presence of cyanide.

Isolation of α-Ketobutyric Acid 2,4-Dinitrophenylhydrazone from Cystathionine Cleavage Mixtures

The α-keto acid was isolated from the deproteinized cleavage mixture by precipitation of the 2,4-dinitrophenylhydrazone from aqueous solution by a method similar to that used by Neuberg and Kobel (13) and Krebs (14) for isolations of pyruvic acid and α-ketobutyric acid, respectively.

Eight 10 mg. portions of cystathionine were dissolved in 0.2 ml. of 1 N HCl and 2 ml. of water. The solutions were then neutralized with 0.4 ml. of 0.5 N NaOH, 0.2 ml. of 0.1 M NaCN neutralized to pH 7 was added, and the volume was adjusted to 3 ml. with water. 1 ml. of the enzyme preparation was added to each mixture. The mixtures were incubated under anaerobic conditions at 38° for 20 minutes. Another 1 ml. portion of enzyme was then added and the incubation was continued for an additional 40 minutes. The contents of all the flasks were then combined and poured into a large test-tube containing 1 ml. of 2.5 N HCl. 3.5 ml. of 10 per cent sodium tungstate were added and the volume was adjusted to 50 ml. with water. After being allowed to stand for 4 hours in the refrigerator, the mixture was centrifuged. The supernatant amounted to 42.5 ml.

To 20 ml. of this supernatant were added 5 ml. of an acid solution of 2,4-dinitrophenylhydrazine (6.25 mg. per 1 ml. of 2 N HCl). After the

mixture was cooled for several hours, the precipitate of the 2,4-dinitro-phenylhydrazone was separated by centrifugation and washed with 5 ml. of 0.25 N HCl. The yield of crude material was 20 mg. It was extracted with three 1 ml. portions of boiling ethyl acetate, each extract being filtered to remove inorganic material. The 2,4-dinitrophenylhydrazone crystalliz-ing from the combined ethyl acetate solutions as long needles amounted to 8 mg., m.p. 198–199°.[1] Its percentage composition corresponded to that of the 2,4-dinitrophenylhydrazone of α-ketobutyric acid.

$C_{10}H_{10}O_6N_4$.   Calculated.   C 42.56, H 3.57, N 19 85
282.2   Found.   " 42.44, " 3 59, " 19.93

An authentic sample of α-ketobutyric acid was prepared according to the method of Tschelinzeff and Schmidt (15). The 2,4-dinitrophenylhydra-zone of the latter compound melted at 196–198°. The melting point of a mixture of the material isolated from the cystathionine cleavage with this authentic α-ketobutyric acid 2,4-dinitrophenylhydrazone showed no de-pression, m.p. 196–198°.

Incubation of Homoserine and Related Compounds with Liver Enzyme Preparation

DL-Homoserine and DL-homoserine lactone were prepared by the method of Livak and coworkers (16). These two compounds and L-homoserine[2] were incubated anaerobically at 38° in water with the liver enzyme prep-aration under a variety of conditions. The amount of α-keto acid for-mation was determined by the same method as that used on the cysta-thionine cleavage mixtures. The cleavage conditions and the yields of α-keto acid from these incubations are summarized in Table II.

Incubation of DL-α-aminobutyric acid, DL-threonine, or DL-homocysteine with the enzyme preparation gave no α-keto acid.

Isolation of α-Ketobutyric Acid 2,4-Dinitrophenylhydrazone from Homoserine Incubation Mixture

A solution of 420 μM (50 mg.) of DL-homoserine in 1 ml. of water and 0.2 ml. of 0.1 M NaCN was incubated anaerobically at 38° for 2.5 hours with 4 ml. of liver enzyme preparation. The pH of the mixture was adjusted to 2 by addition of 2.5 N HCl and the proteins were precipitated by addition of 0.7 ml. of 10 per cent sodium tungstate and separated by centrifugation. The supernatant contained about 150 μM of keto acid. This was treated with 5 ml. of an acid solution of 2,4-dinitrophenylhydrazine (6.25 mg. per

[1] All the melting points were determined on the hot stage and have been cor-rected.
[2] The sample of L-homoserine was kindly supplied by Dr. Marvin D. Armstrong.

1 ml. of 2 N HCl). The 2,4-dinitrophenylhydrazone formed immediately. After having stood for several hours in the refrigerator, the precipitate was

$C_{10}H_{10}O_6N_4$. Calculated. C 42.56, H 3.57, N 19.85
282.2 Found. " 42.55, " 3.87, " 19.71

separated by centrifugation and washed with 0.25 N HCl (yield, 44.5 mg.). The material was extracted with five 1 ml. portions of ethyl acetate. The combined extracts were filtered, concentrated to a volume of 3 ml., and cooled. 20 mg. of the 2,4-dinitrophenylhydrazone separated as yellow needles, m.p. 196–198°. In percentage composition and in melting point

TABLE II

Formation of α-Keto Acid from Homoserine by Incubation with Liver Enzyme Preparation

Substrate	Amount of substrate	Amount of enzyme preparation	Added NaCN (0.004 M)	Incubation time	α-Keto acid	
					Amount	Theoretical amount
	μM	ml.		hrs.	μM	per cent
DL-Homoserine..	13.5	1	+	0.75	3.8	28
"	22.5	0.5	–	1	3.5	16
"	27	2	+	1.75	2.6	10
"	27	1	+	2	3.3	12
"	42	1	+	1.5	13.8	33
"	42	1	–	1.5	19.1	45
"	42	1	–	1.5	19.8	47
"	42	1	–	1	18.5	44
"	54	2	+	0.75	15.7	29
"	84	2	+	1.5	30.5	36
"	420	4	+	2.5	170	40
L-Homoserine . . .	13.5	2	+	1.75	2.0	15
DL-Homoserine lactone .	13.5	1	+	1.5	4.5	33

this substance corresponded to the 2,4-dinitrophenylhydrazone of α-keto-butyric acid.

Admixture of this derivative with an authentic sample caused no depression in the melting point, 197–198°. Also, a mixture of the keto-butyric acid derivative isolated from the homoserine incubation mixture with that obtained from the cystathionine cleavage melted at 198–199°.

Further substantiation of the identity of the α-ketobutyric acid 2,4-dinitrophenylhydrazone derived from homoserine with authentic α-keto-butyric acid 2,4-dinitrophenylhydrazone was obtained by comparison of their infra-red absorption spectra.

The authors extend their appreciation to Dr. Julian R. Rachele for determination and comparison of infra-red absorption spectra and to Mrs. Josephine T. Marshall for carrying out the microanalyses.

SUMMARY

In addition to the formation of cysteine, the formation of an α-keto acid has been detected in the cleavage mixture from the incubation of L-cystathionine with a rat liver enzyme preparation. This keto acid has been identified as α-ketobutyric acid by isolation from the cleavage mixture as the 2,4-dinitrophenylhydrazone. DL-Homoserine was also converted to α-ketobutyric acid by the action of the enzyme preparation under the conditions of cystathionine cleavage. DL-α-Aminobutyric acid did not yield any α-keto acid upon incubation with the enzyme preparation.

BIBLIOGRAPHY

1. Binkley, F., Anslow, W. P., Jr., and du Vigneaud, V., *J. Biol. Chem.*, **143**, 559 (1942).
2. Binkley, F., *J. Biol. Chem.*, **155**, 39 (1944)
3. Teas, H. J., Horowitz, N. H., and Fling, M., *J. Biol. Chem.*, **172**, 651 (1948).
4. Horowitz, N. H., *J. Biol. Chem.*, **171**, 255 (1947).
5. du Vigneaud, V., Brown, G. B., and Chandler, J. P., *J. Biol. Chem.*, **143**, 59 (1942).
6. Armstrong, J. D., and Binkley, F., *J. Biol. Chem.*, **174**, 889 (1948)
7. Dent, C. E., *Biochem. J.*, **40**, p. xliv (1946).
8. Dent, C. E., *Science*, **105**, 335 (1947).
9. Fromageot, C., and Clauser, H., *Biochim. et biophys acta*, **1**, 449 (1947).
10. Smythe, C. V., *J. Biol. Chem.*, **142**, 387 (1942).
11. Sullivan, M. X., *Pub. Health Rep., U. S. P. H. S.*, **44**, 1421 (1929)
12. Friedemann, T. E., and Haugen, G. E., *J. Biol. Chem.*, **147**, 415 (1943).
13. Neuberg, C., and Kobel, M., *Biochem. Z.*, **219**, 490 (1930).
14. Krebs, H. A., *Z. physiol. Chem.*, **217**, 191 (1933).
15. Tschelinzeff, W., and Schmidt, W., *Ber. chem. Ges.*, **62 B**, 2210 (1929).
16. Livak, J. E., Britton, E. C., VanderWeele, J. C, and Murray, M. F., *J. Am. Chem. Soc.*, **67**, 2218 (1945).

THE EFFECT OF ENZYMATIC HYDROLYSIS ON THE NUTRITIVE VALUE OF CASEIN

III. INTRAVENOUS FEEDING IN DOGS*

By JAMES B. ALLISON, BACON F. CHOW, JOHN I. WHITE, AND JAY S. ROTH

(From the Bureau of Biological Research, Rutgers University, and from the Division of Protein Chemistry, of The Squibb Institute for Medical Research, New Brunswick)

Studies by Christensen *et al.* (1, 2) and by Allison and associates (3) have demonstrated that the urinary excretion of peptides is greater than the urinary excretion of free amino acids following the infusion of partial hydrolysates of casein or of fibrin. Christensen and coworkers have shown also that infusion of these hydrolysates results in a greater persistence of peptides in the plasma than of free amino acids. Their results were interpreted to mean that in a given hydrolysate peptides are not as well or as quickly utilized as free amino acids, although some peptides may be more readily utilized than others (2). The following studies were planned to determine the effects of varying the degree of hydrolysis of casein by pancreatic enzymes from 18 to 40 to 60 per cent, on the excretion of peptides in the urine, and on the retention of nitrogen in the animal. These enzymatic hydrolysates were found to have approximately the same contents of the ten essential amino acids and they, therefore, differed from one another only in the proportion of free amino acids and polypeptides. Enzymatic hydrolysis was used so that the destructive forces of other forms of hydrolysis on certain amino acids would be minimized.

Methods

Three adult litter mate beagles were used in these studies. The nitrogen balance indexes of intravenously fed protein hydrolysates were determined by the following equation,

$$UN = (1-K)(AN) + UN_0 \qquad (1)$$

where UN is the urinary nitrogen during the hydrolysate feeding period, K is the nitrogen balance index, AN is the absorbed nitrogen, and UN_0 is the urinary nitrogen excretion in the dog near nitrogen equilibrium on a lactalbumin diet. UN and UN_0 are determined experimentally, AN

equals the amount of hydrolysate nitrogen infused, and K can be calculated from Equation 1. The index (K) is a function of the amount of absorbed nitrogen retained in the body of the animal and may be defined as the rate at which the protein stores of the body are being filled (4).

The normal, adult, parasite-free dogs were placed on a lactalbumin diet at a nitrogen intake of 120 mg. of protein nitrogen per kilo of body weight per day and at an intake of 70 calories per kilo of "probable weight" as calculated from Cowgill's data (5). The dogs received this diet for an adjustment period of 4 days (Days 1 to 4) during which no collections were made. The following 3 days (Days 5 to 7) the dogs were fed the same lactalbumin and the urine was collected and pooled for the determination of UN_0 Sample 1. The hydrolysate was fed intravenously on Days 8 to 11, the dogs receiving 120 mg. of hydrolysate nitrogen per day per kilo of body weight at a rate of 1 mg. of N per minute per kilo of body weight. Immediately after each intravenous feeding the dogs were fed orally a protein-free diet (70 calories per kilo of body weight per day). The urine was collected and analyzed daily for the determination of UN during this infusion period. The infusion was followed by another 3 day (Days 12 to 14) lactalbumin feeding period (120 mg. of nitrogen per kilo per day) for the determination of UN_0 Sample 2.

The daily K values for the 4 days of intravenous feeding were calculated from Equation 1. UN_0 Sample 1 determined immediately before a period of infusion was used for the calculation of K during the first 2 days of intravenous feeding. UN_0 Sample 2 of the lactalbumin feeding period immediately following the infusion period was used to calculate K for the last 2 days of the hydrolysate feeding period.

The free and "bound" α-amino nitrogen concentrations were determined in 24 hour urine samples by the ninhydrin method of Van Slyke, MacFadyen, and Hamilton (6). The bound amino nitrogen was calculated by subtracting the free α-amino nitrogen from the total α-amino nitrogen determined after the urine had been hydrolyzed with an equal part of 12 N HCl for 6 hours at 120° and 15 pounds pressure. The casein hydrolysates were prepared enzymatically so that 18, 40, and 60 per cent of the amino acids were liberated. The methods of preparation have been published in detail elsewhere (7). Only solutions free of pyrogen were used in this study.

Results

The value for UN_0 in Equation 1 should equal the excretion of urinary nitrogen when the dogs are receiving a protein-free diet, the so called excretion of endogenous or body nitrogen. The value for UN_0 may also be determined by feeding a protein with a nitrogen balance index of unity.

The lactalbumin used in these experiments had an average nitrogen balance index of unity in dogs. Thus this lactalbumin was fed to litter mate dogs to determine UN_0 in Equation 1, preventing the protein-depleting effects of feeding a protein-free diet. The average indexes for the 18, 40, and 60 per cent hydrolysates fed intravenously to the dogs are recorded in Table I.

TABLE I

Average Daily Urinary Nitrogen Excretion and Nitrogen Balance Indexes

The determinations were made on three dogs fed intravenously 120 mg. of casein hydrolysate nitrogen per day per kilo of body weight.

Degree of hydrolysis	No. of daily determinations	UN	UN₀	K
per cent		*mg. per day per kg.*	*mg. per day per kg.*	
18	12	158	100	0.52 ± 0.08*
40	12	165	105	0.50 ± 0.08
60	8	150	99	0.57 ± 0.09

* Standard error.

TABLE II

Daily Urinary Amino Nitrogen during Feeding of Lactalbumin Orally and Casein Hydrolysates Intravenously

120 mg. of nitrogen per day per kilo of body weight; average data obtained on three dogs. All values are expressed as mg. per day per kilo.

Nitrogen source	Urinary amino nitrogen					
	18 per cent hydrolysate		40 per cent hydrolysate		60 per cent hydrolysate	
	Free	Bound	Free	Bound	Free	Bound
Lactalbumin.	1.5	4.6	1.3	44.4	1.7	6.9
Hydrolysate	1.3	10.0	2.6	22.4	2.0	17.0
"	2.5	14.9	1.8	19.9	3.0	16.4
"	1.3	17.8	1.9	20.2	2.6	17.7
"	2.1	15.1	2.1	19.7	2.3	22.9
Lactalbumin	1.2	4.6	1.5	3.8	1.6	5.8

These data demonstrate that the retention of nitrogen as measured by the index is the same for the three hydrolysates. The results can be interpreted to mean that approximately half of the nitrogen infused is retained, some of which originated from polypeptide nitrogen in the hydrolysate. Thus at least a part of the peptides of casein as well as the free amino acids may be utilized to build up the nitrogen stores of the

body. Further studies are under way to compare the rate of metabolism of the peptides with free amino acids and to determine the effect of loss of varying amounts of peptides in the urine on the indexes. Preliminary data indicate that both the rate of utilization and loss of peptides in the urine may alter the retention of nitrogen.

The data recorded in Table II illustrate the spilling of free and bound α-amino nitrogen in the urine during the days of infusion of the hydrolysates recorded in Table I. The free α-amino nitrogen excreted daily in
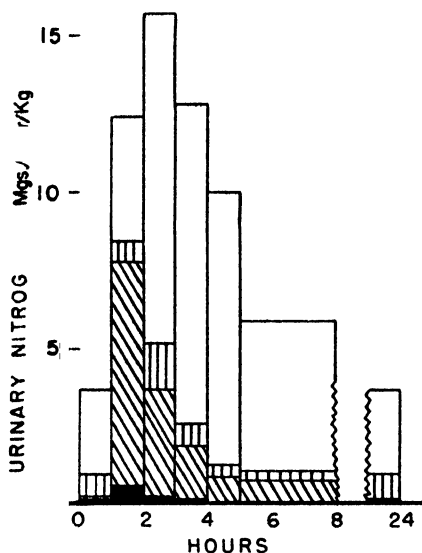
Fig. 1. The rate of urinary nitrogen excretion plotted against time in hours. The data for the 1st hour represent the control. 120 mg. of the 60 per cent hydrolysate N per kilo of body weight were infused during Hour 1 to 2.   The solid blocks represent the excretion of α-amino nitrogen, the blocks with slanted lines, the rate of excretion of peptide nitrogen, the blocks with vertical lines the rate of excretion of ammonia nitrogen, and the clear blocks the rate of excretion of urea nitrogen.

the urine was not increased very much above the lactalbumin control during intravenous feeding, a result which is in agreement with data previously published by Allison, Seeley, and Ferguson (3) who pointed out that excretion of free amino acids had little effect on the index of protein hydrolysates. The bound or peptide nitrogen was increased significantly, irrespective of the degree of hydrolysis. These data demonstrate that there is a fraction of bound nitrogen which is lost in the urine, possibly because it is not readily utilized by the animal, or it is excreted before it can enter into the metabolism of the body. That most of the peptide nitrogen is lost during the hour of infusion and shortly thereafter is il-

lustrated by the data obtained on the 60 per cent hydrolysate in Fig. 1. The first series of blocks (Hour 0 to 1) in Fig. 1 represents the excretion of various forms of urinary nitrogen under control conditions. The second series of blocks illustrates the excretion during the hour of infusion (Hour 1 to 2), while the remainder of the blocks record data obtained after the infusion had ended. The excretion of peptide nitrogen was similar in the 18, 40, and 60 per cent hydrolysate. Sufficient peptide nitrogen is lost in the urine, therefore, to reduce the nitrogen balance index, but the effect on the index is independent of the degree of hydrolysis.

The increases in excretion of α-amino nitrogen, ammonia nitrogen, and urea nitrogen are also illustrated in Fig. 1. The increase in excretion of α-amino nitrogen and ammonia was slight but greatest during and immediately following the infusion. The increased excretion of urea was built up during the hours following infusion, reaching a maximum for most of the hydrolysate at 3 to 4 hours, returning gradually to control values over a period of 10 or more hours (3). Preliminary data indicate that the return to the control value is slower in the 18 and 40 per cent than in the 60 per cent hydrolysate, results which suggest a less rapid catabolism of peptides than of amino acids. This suggestion is being investigated further.

SUMMARY

The nitrogen balance index for 18, 40, and 60 per cent enzymatic hydrolysates is approximately 0.5 and independent of the degree of hydrolysis. Both free and bound amino nitrogen contribute to the retention of nitrogen in the animal. Urinary α-amino nitrogen increases slightly during and immediately following the infusion of these hydrolysates. The excretion of peptide nitrogen increases similarly but more markedly, with no significant effect of the degree of hydrolysis on this excretion. The excretion of urinary ammonia nitrogen increases slightly during the hour of infusion and the hour or two following it, while urea nitrogen rises gradually to a maximum at 3 or 4 hours, returning to control values 10 or more hours after infusion.

BIBLIOGRAPHY

1. Christensen, H. N., Lynch, E. L., and Powers, J. H., *J. Biol. Chem.*, **166**, 649 (1946).
2. Christensen, H. N., Lynch, E. L., Decker, D. G., and Powers, J. H., *J. Clin. Invest.*, **26**, 849 (1947).
3. Allison, J. B., Seeley, R. D., and Ferguson, F. P., *J. Biol. Chem.*, **171**, 91 (1947).
4. Allison, J. B., *Am. J. Med.*, **5**, 419 (1948).
5. Cowgill, G. R., *Am. J. Physiol.*, **85**, 45 (1928).
6. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, **150**, 251 (1943).
7. Chow, B. F., Allison, J. B., and White, J. I., *J. Nutr.*, **36**, 231 (1948).

PHOTOLYTIC AND ENZYMATIC TRANSFORMATIONS
OF PTEROYLGLUTAMIC ACID

By OLIVER H. LOWRY, OTTO A. BESSEY, and ELIZABETH J. CRAWFORD

(*From the Department of Pharmacology, Washington University School of Medicine, St. Louis, the Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, and the Division of Nutrition and Physiology, The Public Health Research Institute of The City of New York, Inc., New York*)

Bloom *et al.* (1) found that ultraviolet light converted pteroylglutamic acid (PGA) which is not fluorescent into a fluorescent material. Stokstad, Fordham, and de Grunigen (2) observed that sunlight caused the liberation of a diazotizable amine, indicating that cleavage had occurred between the pterine and the *p*-aminobenzoylglutamic acid components. As part of a search for a sensitive analytical reaction for PGA, this photolytic effect has been further investigated, and a rather interesting sequence of transformations has been found to occur. Evidence to be presented below indicates that when PGA (I) is irradiated with ultraviolet light it is first converted to 2-amino-4-hydroxy-6-formylpteridine (II) and a diazotizable amine. With further irradiation the aldehyde is converted to the corresponding 6-carboxylic acid derivative (III) and finally to the decarboxylated 2-amino-4-hydroxypteridine (AHP) (IV). This, in turn, is susceptible to oxidation to isoxanthopterin (V) by an enzyme present in cream, which is probably identical with xanthine oxidase and xanthopterin oxidase.
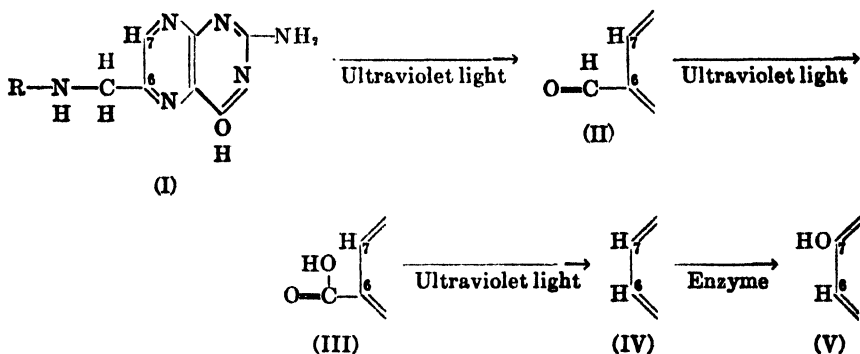
It seems possible that either the increase in fluorescence on irradiation or the more specific change in fluorescence resulting from the enzymatic oxidation of the final irradiation product might prove analytically useful. The aldehyde formed as the first photolytic product has proved to be a

remarkably effective inhibitor of the oxidative enzyme.   As little as 2 ×
10^{-4} γ per ml. causes definite inhibition.

It was observed that a solution of 20 γ of PGA per ml. in 0.01 N acetic
acid became very fluorescent (Table I) when irradiated with a source of
the 365 mμ mercury line (a General Electric B-H4 lamp).[2]   The develop-
ment of fluorescence was accompanied by the appearance of diazotizable
amine with a molar yield of about 80 per cent (compared to p-aminobenzoic
acid).[3]   When this fluorescent solution was diluted 4-fold with 0.05 N
acetic acid and subjected to more intense irradiation (General Electric
A-H4 or A-H5 lamp), there was little change in fluorescence as measured
in borate buffer at pH 9, but the fluorescence in 0.1 M phosphate buffer
of pH 6.8 fell appreciably, suggesting that there had been further change
(Table I, lower section).   The occurrence of such change could be con-
firmed by treating the irradiated solutions with a preparation of "xanthine
oxidase" from cream.   This enzyme was without effect on the products
of mild irradiation, but when incubated with the products of more intense
irradiation, the enzyme effected a large increase in the fluorescence in
phosphate buffer (Table I, Column 5).   The enzyme concentrate was pre-
pared according to Ball (4).   The question of its identity with xanthine
oxidase or the xanthopterin oxidase of Wieland and Liebig (5) and Kalckar
and Klenow (6) will be discussed in a separate paper (7).

Thus two distinct fluorescent irradiation products appeared to be formed
from PGA.   That still a third substance was produced early in the reaction
was indicated by the presence of material inhibitory to the above enzyme.
This inhibitory substance appeared and then disappeared before the en-
zyme-sensitive product was formed (Table I, Column 7).

[1] This study was greatly simplified by having available all of the 6-substituted
derivatives of 2-amino-4-hydroxypteridine. The PGA, pteroyldiglutamic acid,
pteroyltriglutamic acid, 2-amino-4-hydroxy-6-formylpteridine, and 2-amino-
4-hydroxy-6-carboxypteridine were generously furnished by Dr. E. L. R. Stokstad
and Dr. T. H. Jukes of the Lederle Laboratories Division, American Cyanamid Com-
pany. The 2-amino-4-hydroxy-6-hydroxymethylpteridine, 2-amino-4-hydroxypteri-
dine, xanthopterin, and isoxanthopterin were kindly supplied by Dr. G. H. Hitchings
of Burroughs Wellcome and Company.

[2] The fluorescence was measured with either the Coleman fluorometer model 12
or the very sensitive Farrand micro fluorometer (3). The light filter combination
used consisted of a primary of Corning glass 5860 (transmits Hg line 365 mμ), and a
secondary of Corning glass 4308, Wratten gelatin film 2A, and Corning glass 3389
in this order receding from the sample tube. This is similar to the filter combination
usually employed with thiochrome, but is modified to reduce the optical blank (3).

[3] The diazotizable amine disappears completely (Table I) when irradiated with the
powerful A-H5 lamp which has a clear glass envelope and emits some light at wave-
lengths shorter than 365 mμ.

It seemed worth while to explore the possibility that all three of these products were 2-amino-4-hydroxypteridines, substituted in the 6 position. The 6-methyl and the 6-hydroxymethyl derivatives were ruled out as major products, since they were both stable to ultraviolet irradiation and were unaffected by the cream enzyme. Xanthopterin (the 6-hydroxy

TABLE I

Effect of Ultraviolet Irradiation on Pteroylglutamic Acid

Time of irradiation (1)	Fluorescence, galvanometer divisions			Change due to enzyme (5)	Diazotizable amine (6)	Enzyme inhibitor (7)
	Before enzyme		After* enzyme			
	Borate, pH 9 (2)	Phosphate, pH 6.8 (3)	Phosphate, pH 6.8 (4)			
Irradiation 2 in. from arc of B-H4† lamp (in 0.01 N acetic acid)						
min.					*per cent theoretical*	*per cent theoretical‡*
0	1	0	1	1	2	1.6
15	12	5	5	0	25	14
30	52	27	26	−1	78	29
45	60	30	30	0	78	7
60	66	33	34	1	78	0.3
120	67	32	36	4	78	0.3
Irradiation continued 2.8 in. from arc of A-H5† lamp						
15	66	28	51	23	40§	
30	67	25	60	35	22§	
60	66	20	73	53	4§	
180	63	17	69	52		

* Enzyme treatment after irradiation.

† General Electric Company mercury arc lamps. The 100 watt B-H4 lamp has a filter jacket which passes primarily the 365 mμ line. The 250 watt A-H5 lamp has a clear glass jacket, and hence passes wave-lengths down to about 300 mμ.

‡ Calculated as per cent of the possible 6-aldehyde which could be formed from the PGA present. The inhibitor was measured by its ability to inhibit the enzymatic oxidation of 2-amino-4-hydroxypteridine, and was compared with known amounts of 6-aldehyde (7).

§ Interpolated from a separate experiment.

derivative) could be eliminated by its behavior with the cream enzyme. This oxidase converts xanthopterin into leucopterin (5, 6) which, instead of being more fluorescent, is non-fluorescent in the neutral pH range. This left three other possibilities, the 6-aldehyde, the 6-carboxylic acid, and the 6-hydrogen derivative or AHP. These compounds proved to have the properties of the three successive photolytic products of PGA. Thus

the aldehyde is a strong inhibitor of the cream enzyme, and both the alde-
hyde and the carboxylic acid are converted by irradiation into AHP, which
was in turn found to be the only member of the series readily oxidized by
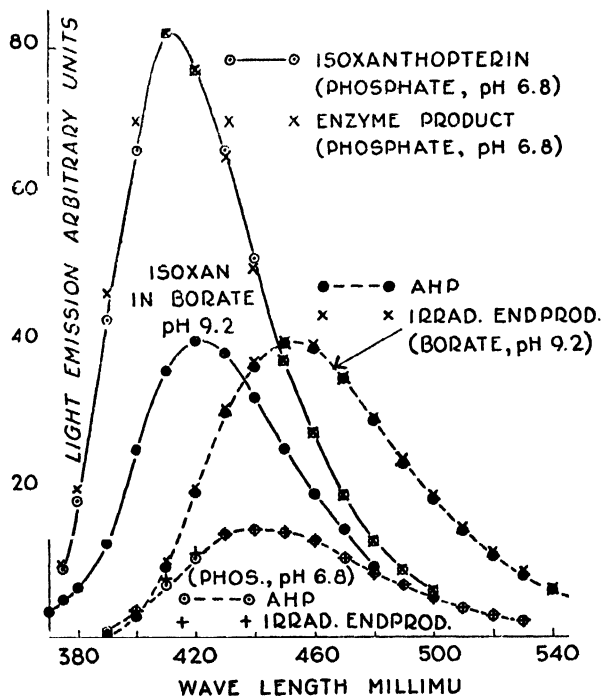the cream enzyme. More complete evidence for the identity of these
materials follows.

Fig. 1. Fluorescence spectra of known and suspected 2-amino-4-hydroxypteridine
(AHP) and isoxanthopterin. The irradiation end-product is the third successive
compound resulting from ultraviolet irradiation of pteroylglutamic acid. The
enzyme product is the substance formed when "xanthine oxidase" is allowed to
act on this irradiation end-product.

The product of enzyme action on the end-product of photolysis of PGA
appears to be isoxanthopterin, i.e. 2-amino-4,7-dihydroxypteridine. Its
fluorescence spectrum[4] (Fig. 1) coincides with that of synthetic isoxanthop-

terin.   None of the other pteridines of this series have fluorescence spectra
in this region.   The fluorescence spectrum of synthetic AHP similarly was
found to coincide with the product of intense irradiation of PGA (Fig. 1).
This agreement, however, could not alone identify the compound, (1) since
some of the other pteridines have fluorescent spectra which are very similar
and (2) the 6-methyl and 6-hydroxymethyl analogues show a similar quan-
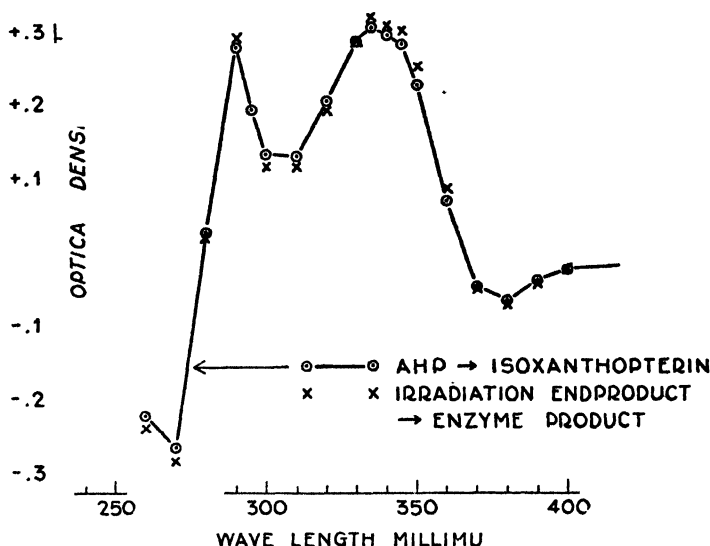titative change between phosphate and borate buffers.

Fig. 2. Change in absorption spectra of known and suspected AHP with enzyme
treatment.   A solution containing 8.8 γ per ml. of 2-amino-4-hydroxypteridine
(AHP) was converted to isoxanthopterin with "xanthine oxidase;" the resultant
change in the absorption spectrum is recorded.   Similarly, the end-product of ir-
radiation of pteroylglutamic acid was treated with the same enzyme; the compa-
rable difference spectrum is given.

Stronger evidence is furnished by the change in the absorption spectra
(Fig. 2) which occurred when synthetic AHP and presumptive AHP (from
irradiation) were treated with cream enzyme in the Beckman spectro-
photometer.   The concordance of the two difference spectra could scarcely
be fortuitous.   The difference spectra are given rather than the spectra
themselves, since the irradiated specimen contained ultraviolet-absorbing
degradation products of p-aminobenzoylglutamic acid, as well as the pteri-
dine.   The actual absorption spectrum obtained after treatment of known
AHP with enzyme agrees with that of known isoxanthopterin.   There
seemed to be little question left that the final irradiation product was AHP,
and that the enzyme product was isoxanthopterin.

All the evidence indicated that the precursor of AHP was 2-amino-4-

hydroxy-6-carboxypteridine. The precursor could be formed by irradiating a solution of PGA for several hours with a B-H4 lamp (Table I, upper section). Such a solution was neither very inhibitory for the cream enzyme, nor did it contain appreciable enzyme substrate. That is, the fluorescent material present was neither the 6-aldehyde nor AHP. The precursor had the same distribution coefficient between water and organic solvents as known 6-carboxylic acid. Both substances were convertible into AHP at identical rates by irradiation. The changes in absorption spectrum resulting from this further irradiation were quite similar for both known 6-carboxylic acid and for presumptive 6-carboxylic acid from irradiated PGA. The two difference spectra did not coincide exactly because of concomitant change in the p-aminobenzoylglutamic acid present in the sample from irradiated PGA.[3] A small amount of suspected 6-carboxylic acid was obtained by differential elution of a sample of irradiated pteroyltriglutamic acid from Florisil, and the absorption spectrum concurred with that of known 6-carboxylic acid.

The precursor of 6-carboxylic acid, and apparently the first fission product of PGA, is almost certainly 2-amino-4-hydroxy-6-formylpteridine. At low substrate concentrations (10^{-6} to 10^{-7} M), oxidation of AHP to isoxanthopterin is markedly inhibited by less than 10^{-3} γ of 6-aldehyde per ml. (7) or by an equal amount of presumptive aldehyde from short irradiation of PGA. No other inhibitory substance with activity approaching this potency has been found. A small amount of this early irradiation product was isolated from a strong solution of irradiated PGA (1 mg. per ml.). The amount of dissolved oxygen was apparently inadequate for the complete oxidation of such a strong solution, and as a result there was an accumulation of the aldehyde. Comparison of the absorption spectrum of this material with that of known 6-aldehyde (Fig. 3) confirmed the identity of this initial photolytic product.

The three pteridine photolytic products from PGA and the enzymatic derivative of the last of these would seem to be reasonably well identified. Perhaps the strongest evidence is furnished by the chain of reactions as a whole. One may start with any member of the series and obtain in the predicted manner the next members of the sequence.

Factors Affecting Photolysis—The oxidative photolysis, as expected, proceeds very slowly in the complete absence of dissolved oxygen. The slow reaction which does occur is presumably made possible by photolytic release of oxygen from the water.

The rate of reaction is maximal at pH 3, although it has been carried to completion both at pH 1 and pH 7. At pH 9 the reaction has not been studied thoroughly. Fluorescence develops, but much more slowly than in the acid range.

Fluorescence does not appear if samples are irradiated in the presence

of alcohols.   This is apparently not due to inhibition of fission, but due to a light-catalyzed reaction between the alcohols and the liberated pteridines. The fluorescence of all three pteridines concerned is greatly decreased if they are irradiated for a short time in the presence of a 1 per cent aqueous solution of benzyl alcohol.   Methyl, ethyl, butyl, and benzyl alcohol, in this order, show increasing capacity to inhibit the appearance of fluorescence on irradiation of PGA.   This phenomenon would need to be considered in any attempt to use the above sequence of reactions for analytical purposes.
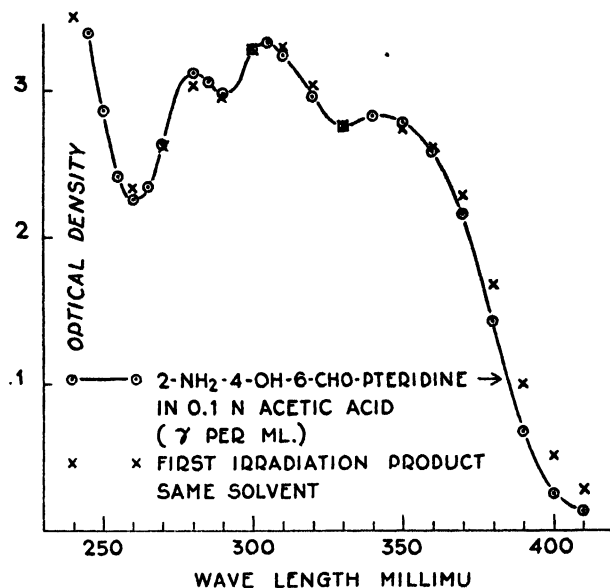
FIG. 3. Absorption spectra 2-amino-4-hydroxy-6-formylpteridine and first irradiation product of pteroylglutamic acid.

Fluorescent Properties of Pteridines—The pteridines are fluorescent over a wide pH range, but the fluorescence is markedly influenced by the composition of the medium.   Thus the amount and character of emitted light vary markedly with pH (Fig. 1) (see also Jacobson and Simpson (9)). Particularly striking is the degree to which a number of anions quench the fluorescence of pteridines (Figs. 4 and 5).   The fluorescence of AHP or xanthopterin is 90 to 95 per cent quenched in 0.3 M phosphate at pH 6.8 (Fig. 4).   Isoxanthopterin is much less affected.   For this reason the conversion of AHP to isoxanthopterin is accompanied by a large increase in fluorescence if the reaction takes place in 0.1 M or stronger phosphate buffer or in certain other buffers (Fig. 5).   In dilute salt solutions AHP and xanthopterin differ only a little in the intensity of their fluorescence.   Since there is a linear relationship between the reciprocal of the fluorescence and,
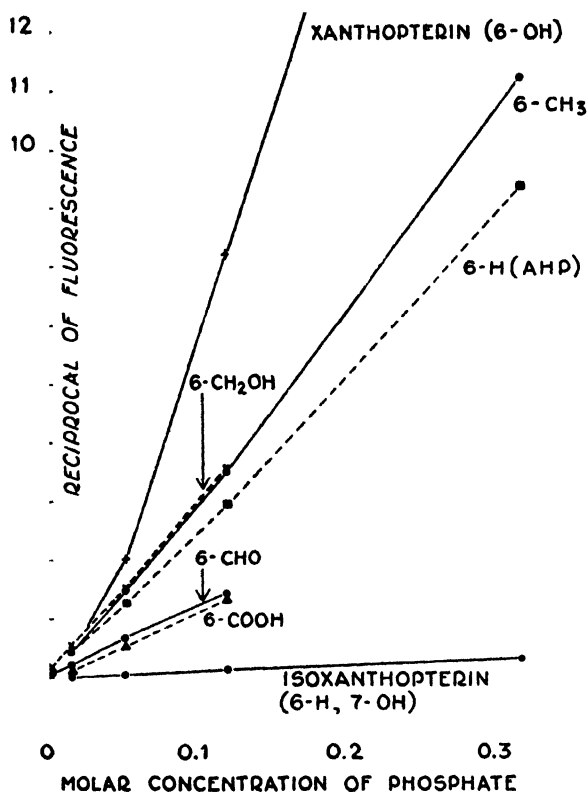
FIG. 4. Quenching of fluorescence of pterines by phosphate. The compounds represented are all 2-amino-4-hydroxypteridines with the indicated substituents in the 6 or 7 position. Reciprocal of fluorescence = (fluorescence without salt)/(actual fluorescence).

the concentration of the quenching anion (Figs. 4 and 5), it seems probable that the quenching can be ascribed to the formation of a dissociable non-fluorescent combination between the particular ion and the pteridine.[5]

[5] If anion (A) + pterine $(P) \rightleftarrows AP$, then

$$\frac{(A)(P)}{(AP)} = K$$

$$A = \frac{K(AP)}{(P)} \quad K\left[\frac{\text{(total pterine)} - (P)}{(P)}\right] = K\left[\frac{\text{(total pterine)}}{(P)} - 1\right]$$

If P alone is fluorescent then

$$(A) \quad K\left[\frac{\text{fluorescence without salt}}{\text{actual fluorescence}} - 1\right]$$

When fluorescence = 50 per cent, $A = K$. The amount of bound anion is ignored in the above equation, since it is negligible.

The apparent dissociation constant of this complex is given by the salt concentration necessary to reduce the fluorescence to 50 per cent, *e.g.* 0.043 mole of acetate per liter for AHP. Weil-Malherbe has discussed a similar situation with other fluorescent materials (10). When xanthopterin solutions are diluted with phosphate buffer, the fluorescence does not immediately reach a stable value, but falls somewhat for a period of minutes.

The quenching by anions is much less marked at more alkaline pH values (7.5 or greater), but the fluorescence of certain pteridines, notably that of xanthopterin, is enhanced at slightly alkaline pH by a variety of di- and trivalent cations, in some cases at very low cation concentrations.
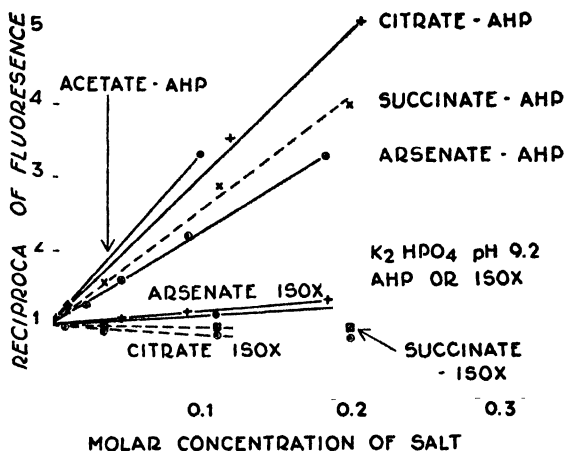

Fig. 5. Quenching of the fluorescence of 2-amino-4-hydroxypteridine (AHP) and isoxanthopterin (ISOX) by various anions. Reciprocal of fluorescence = (fluorescence without salt)/(actual fluorescence). Unless indicated, the pH is approximately 7.

Because of the marked susceptibility of the fluorescence of the pteridines to quenching or enhancement, fluorescent measurements with the pteridines must be controlled with internal standards if quantitative concentration values are desired.

SUMMARY

It has been observed that, when pteroylglutamic acid is irradiated with ultraviolet light in slightly acid solution, it undergoes oxidative cleavage to yield in succession 2-amino-4-hydroxy-6-formylpteridine, 2-amino-4-hydroxy-6-carboxypteridine, and 2-amino-4-hydroxypteridine. The final photolytic product is susceptible to oxidation to isoxanthopterin by "xanthine oxidase" from cream. The four compounds were identified chiefly

by their fluorescence spectra, absorption spectra, and behavior toward xanthine oxidase. The aldehyde, which is the first photolytic product, is an extremely active inhibitor of xanthine oxidase. As little as 2×10^{-4} γ per ml. produces demonstrable inhibition.

BIBLIOGRAPHY

1. Bloom, E. S., Vandenbelt, J M., Binkley, S. B., O'Dell, B. L., and Pfiffner, J. J., *Science*, **100**, 295 (1944).
2. Stokstad, E. L. R., Fordham, D., and de Grunigen, A., *J. Biol. Chem.*, **167**, 877 (1947).
3. Lowry, O. H., *J. Biol. Chem.*, **173**, 677 (1948).
4. Ball, E. G., *J. Biol Chem.*, **128**, 51 (1939).
5. Wieland, H., and Liebig, R , *Ann. Chem.*, **555**, 146 (1944).
6. Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, **172**, 349 (1948).
7. Lowry, O. H., Bessey, O A., and Crawford, E. J., *J Biol. Chem*, **180**, 399 (1949).
8. Burdett, R. A., and Jones, L. C., Jr., *J. Optical Soc. Am.*, **37**, 551 (1947)
9. Jacobson, W , and Simpson, D. M., *Biochem. J* , **40**, 3 (1946).
10. Weil-Malherbe, H., *Biochem. J.*, **40**, 363 (1946).

PTERINE OXIDASE

By OLIVER H. LOWRY, OTTO A. BESSEY, and ELIZABETH J. CRAWFORD

(From the Department of Pharmacology, Washington University School of Medicine,
St. Louis, the Department of Biological Chemistry, University of Illinois
College of Medicine, Chicago, and the Division of Nutrition and Physiology, The
Public Health Research Institute of The City of New York, Inc., New York)

When pteroylglutamic acid (PGA) is irradiated with ultraviolet light,
oxidative cleavage occurs and three pteridines are formed in succession (1).
It was observed that the last of the series, 2-amino-4-hydroxypteridine
(AHP), could be oxidized to isoxanthopterin by a concentrate of xanthine
oxidase from cream. It was also observed that the first photolytic product,
2-amino-4-hydroxy-6-formylpteridine, was a remarkably effective in-
hibitor of this enzymatic oxidation. As little as 2×10^{-4} γ of the aldehyde
per ml. produced demonstrable inhibition (1). This enzyme and this in-
hibition have been further explored and it appears that a single enzyme in
cream is responsible for the oxidation of xanthine, xanthopterin, and AHP.
The dissociation constant for the aldehyde-enzyme complex is so small
that it has been possible to estimate an upper limit of the number of equiva-
lents of enzyme present.

Materials and Methods

The 2-amino-4-hydroxypteridine (AHP), xanthopterin, and isoxanthop-
terin were obtained through the courtesy of Dr. G. H. Hitchings of Bur-
roughs Wellcome and Company. The 2-amino-4-hydroxy-6-formylpteri-
dine was kindly furnished by Dr. T. H. Jukes of the Lederle Laboratories
Division, American Cyanamid Company. The oxidase preparations will
be described below.

The enzymatic oxidation of xanthine to uric acid was measured by the
increase in optical density at 295 mμ (Kalckar (2)). For substrate con-
centrations of the order of 10^{-5} M, the enzymatic oxidations of xanthopterin
to leucopterin, and AHP to isoxanthopterin were similarly followed by
measuring the increases in the absorption spectra at 340 mμ (Kalckar and
Klenow (3)) and 335 mμ respectively. For work with higher dilutions of
these two substrates the oxidations were followed fluorometrically. The
fluorescence of xanthopterin disappears in the neutral pH range on con-
version to leucopterin, and may be conveniently followed in the fluorom-
eter (Kalckar and Klenow (3)).

In 0.1 M phosphate or acetate buffer in the pH range from 4 to 7 there is

a 5- to 10-fold increase in fluorescence upon conversion of AHP to isoxanthopterin (1). This increase can be used to measure the oxidation. At pH values more alkaline than 7 the enzymatic change in fluorescence becomes less and is reversed at pH 9. With the Farrand micro fluorometer (4) the oxidation of either xanthopterin or AHP can be followed with substrate concentrations from 10^{-5} down to as low as 3×10^{-8} mole per liter (0.005 γ per ml.). It is thus possible by using both spectrometric and fluorometric means to measure the enzymatic oxidation of xanthopterin or AHP through a 1000-fold range in substrate concentration.

Flavin-adenine dinucleotide (FAD) present in the enzyme was measured by a method detailed elsewhere (5). This method is based on the fact that FAD has only about 10 per cent of the fluorescence of flavin mononucleotide or riboflavin. The flavin was split from the proteins by heating 4 minutes at 100°. The fluorescence of an aliquot was measured, following which an FAD-splitting enzyme from potato (6)[1] was added. The increase in fluorescence, with appropriate internal standards, was used as a measure of the FAD.

Pterine Oxidase—The procedure given by Ball (7) for the preparation of xanthine oxidase was followed closely. The resultant material seemed very comparable to his preparation. The activity toward xanthine appeared to be at least as great, and the ratio of optical density at 280 mμ to that at 450 mμ was 16 as compared to 12, as reported by Ball. This material was used for most of this study. A small sample was further fractionated at 0° with neutralized ammonium sulfate (pH 6.8), as suggested by Kalckar (8). The flavin-adenine dinucleotide was measured in each of five successive fractions. The FAD accounted for more than 98 per cent of the total riboflavin of all the fractions. The protein was estimated from the light absorption at 280 mμ by means of the coefficient determined by Ball (7). The enzyme activity and FAD content maintained a constant ratio in all fractions through a 9-fold change in activity relative to protein (Table I). The most active fraction had properties quite similar to the best preparation of Corran, Dewan, Gordon, and Green (9). They found a ratio of 6.2 between the optical densities at 275 and 450 mμ, which is close to the $D_{280}:D_{450} = 7.8$ for the most active fraction listed in Table I. In this fraction 200,000 gm. of protein were associated with each mole of FAD (calculation from data of Corran *et al.* indicated 170,000 gm. of protein per mole of FAD). The FAD as measured fluorometrically agreed exactly with the optical absorption of the heated extract, but only accounted for about a third of the optical density at 450 mμ of the unheated enzyme solution (Table I, last column). This agrees exactly with Corran *et al.* who

[1] Lowry, O. H., Bessey, O. A., and Love, R. H., in preparation.

measured FAD through its function as coenzyme for D-amino acid oxidase. Part of this apparent absorption may be due to light scattering by the protein; it is also possible that the absorption spectrum of FAD is altered by its combination with protein. The failure to find extra absorption in the heated extract argues against the presence of a second chromogenic prosthetic group.

If the FAD is a direct measure of the number of moles of enzyme present, the turnover number at 30° is about 125 for xanthine at pH 6.8 in 0.1 M phosphate buffer (see below for the means used to measure xanthine oxidation). Evidence to be presented in another section indicates that the

TABLE I

Fractionation of Pterine Oxidase

(NH₄)₂SO₄* fraction	$\frac{D_{290}\dagger}{D_{450}}$	Total‡ protein	Total FAD	Protein FAD	Enzyme activity			$\frac{D_{450}}{D_{450} \text{ calculated from FAD}}$
					Vs. AHP		*Vs.* Xanthine	
					V_{max}§	V_{max}‖	V_{max}‖	
M		*mg.*	10^{-9} *mole*	$\frac{10^5 \text{ gm.}}{\text{per mole}}$				
1.35		11.3	6.0	18.2	2.0	37		
1.48	40.0	8.4	6.9	12.1	3.3	40	128	3.5
1.69	21.6	9.8	15.3	6.4	5.8	37		3.4
1.96	12.4	12.5	38.2	3.3	12.4	41		3.0
2.42	7.8	11.9	58.5	2.0	18.5	37	123	3.0

* The fraction analyzed is that which precipitated between the next lower and the given concentration of ammonium salt (0°, pH 6.8).
† Optical density.
‡ Calculated from D_{280}.
§ Moles of substrate split per minute per 100,000 gm. of protein at 30° at pH 6.8 in 0.1 M phosphate.
‖ Moles of substrate split per minute per mole of flavin-adenine dinucleotide.

number of active molecules or active centers cannot exceed about 60 per cent of the number of FAD molecules in this preparation; hence the turnover number is 210 or more. Corran *et al.* calculated a turnover number of 306 for hypoxanthine at 38°. In what follows the active enzyme will be considered to be numerically equal to 60 per cent of the molecules of FAD.

Activity toward AHP—With sufficient AHP to saturate the enzyme there is only minor dependence of the initial velocity of reaction, V_{max}, on pH over a wide pH range (Fig. 1). The pH optimum is in the neighborhood of 5.5. The Michaelis-Menten constant, K_s, is quite low, varying from 0.5×10^{-6} to 3×10^{-6} M over the pH range measured (Fig. 1). Under a given set of conditions the velocity data fit the equation $K_s = (S)(V_{max}. -$

$V)/V$ over the entire range of substrate concentration (S). (Both V_{max}. and K_s vary somewhat with the kind and amount of salt present. The data (Fig. 1) thus represent the mean values of measurements made under a variety of conditions.) The greater dependence of rate on pH at very low substrate concentrations is also indicated in Fig. 1 $(V_{dil}.)$. The temperature coefficient is 1.04 per degree $(Q_{10} = 1.5)$ in the range 15–45°.

Inhibitors—Several related pterines are competitive inhibitors for the enzymatic oxidation of AHP. Isoxanthopterin, the product of enzymatic oxidation of AHP, has a dissociation constant with the enzyme which is
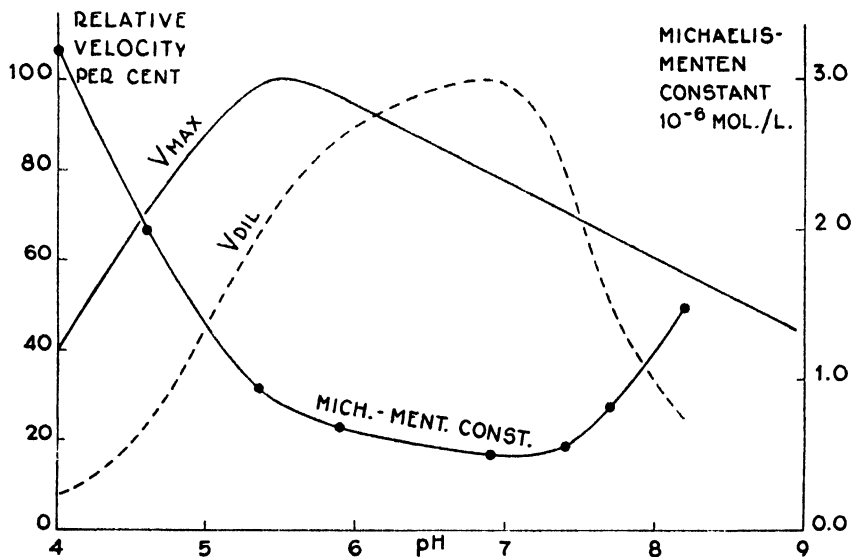
FIG. 1. The effect of pH on the velocity of enzymatic oxidation of 2-amino- 4 hydroxypteridine at saturating concentrations of substrate (V_{max}) and at high dilution of substrate (V_{dil}), and on the Michaelis-Menten constant.

not very different from the K_s of AHP. An interesting consequence of this is that during the oxidation of AHP there is an approximation to a first order reaction not only at high dilution but also at substrate concentrations up to several times the K_s.

The most potent inhibitor found is 2-amino-4-hydroxy-6-formylpteridine (Tables II, III, IV). The inhibitory constant, K_i, is of the order of 10^{-9} M, or 1000 times smaller than the K_s for AHP. Other pterines inhibit, but to a much lesser degree, and it is necessary in each case to rule out the possibility that a trace of the aldehyde might be present. Kalckar *et al.* (10) have given evidence that the inhibition by folic acid of the enzymatic oxidation of xanthine and xanthopterin results from contamination

with a little of the aldehyde. In the present investigation it was similarly found that folic acid inhibits AHP oxidation and that this inhibition is sharply diminished if some of the fluorescent impurities are removed by extraction. Xanthine (1 γ per ml.) causes temporary inhibition of AHP oxidation, until it is itself oxidized by the enzyme. Hofstee has studied a more extensive series of inhibitors for xanthine and xanthopterin oxidation with similar findings (11). No other inhibitor has been found, however, with activity approaching that of the 6-aldehyde.

TABLE II

Inhibition of Cream Enzyme by 2-Amino-4-hydroxy-6-formylpteridine

Substrate, 2-amino-4-hydroxpteridine, 78 $\times$ 10^{-9} mole per liter. All values recorded as 10^{-9} mole per liter, except V which is moles per minute per mole of E_T. $V_{max.}$ = 62 moles per minute per mole of E_T. K_S = 0.71 $\times$ 10^{-6} mole per liter. The symbols are defined in the text.

FAD of enzyme	6-CHO (i_T)	$(E_T)^*$	V	(ES)	(E)	(Ei)	(i)	K_i
9.3	0	5.3	6.0	0.52	4.8			
9.3	1.18	5.3	5.1	0.44	4.0	0.86	0.32	(1.5)†
9.3	2.26	5.3	3.9	0.34	3.14	1.82	0.44	0.8
9.3	5.76	5.3	1.57	0.14	1.26	3.90	1.86	0.6
2.56	0	1.53	6.1	0.153	1.38			
2.56	0.62	1.53	4.3	0.107	0.97	0.45	0.17	(0.4)†
2.56	1.18	1.53	3.5	0.089	0.80	0.64	0.54	0.7
2.56	2.26	1.53	2.19	0.057	0.50	0.97	1.29	0.7
2.56	5.76	1.53	0.85	0.021	0.19	1.32	4.44	0.6

* Assuming moles of enzyme = 0.6 $\times$ moles of FAD present.

† These values are less reliable than the rest due to the very low concentration of aldehyde.

The apparent dissociation constant of the 6-aldehyde-enzyme complex is so small that partial inhibition is observed with amounts of the aldehyde which are lower, on a molar basis, than the enzyme itself. Indeed, with low substrate concentrations, each mole of the 6-aldehyde appeared to inhibit more than 1 mole of enzyme, as measured by its FAD content. For example, in one case (Table II) the concentration of enzyme FAD was 9.3 $\times$ 10^{-9} M, and the substrate (AHP) concentration was 78 $\times$ 10^{-9} M, or only sufficient to keep about 10 per cent of the enzyme combined with substrate; i.e., V was equal to 10 per cent of $V_{max.}$. When the solution was made 2.26 $\times$ 10^{-9} M with respect to the 6-aldehyde inhibitor, the reaction velocity was reduced to 65 per cent of the control. That is, the 2.26 $\times$ 10^{-9} mole per liter of inhibitor had apparently inactivated 35 per cent of the enzyme or 3.3 $\times$ 10^{-9} mole per liter as measured by the FAD. Since

the inhibition is clearly competitive and reversible, the most plausible explanation would seem to be that part of the FAD was not present in active enzymatic combination. The data to follow suggest that only 60 per cent of the FAD is associated with active enzyme. (It would, however, also be reasonably consonant with the data if 2 moles of FAD were associated with each active enzyme molecule or center. Philpot (12), as a result of ultracentrifugal data on the enzyme preparation of Corran *et al.* (9), concluded that 1.4 to 3.1 moles of FAD were associated with each mole of enzyme.) The extremely low dissociation constant of the enzyme-inhibitor complex permits a virtual titration of the enzyme.

In most instances of competitive inhibition, the amount of competitor, i, combined with enzyme, E, is negligible in comparison with the total amount of inhibitor present, and hence in the mass law equation, $((E)(i))/(Ei) = K_i$, it is possible and usual to consider $(i) = (i_T)$, the concentration of total inhibitor (13, 14). In the present instance, however, a substantial proportion of inhibitor is combined with enzyme and cannot be ignored. The following equations serve to evaluate K_i, the enzyme-inhibitor dissociation constant, and the number of moles, n, of FAD (whether active or inactive) which are associated with each mole or equivalent of active enzyme.

Let (E_T), (ES), (Ei), and (E) represent respectively molar or equivalent concentration of total enzyme, enzyme combined with substrate, enzyme combined with inhibitor, and free enzyme. By definition $(FAD) = n(E_T)$, $V_{max.}$ is the velocity of enzyme activity with excess substrate and without inhibitor, and V is the observed velocity with given amounts of substrate and inhibitor. (S) is the substrate concentration. Then

$$(E_T) = (ES) + (Ei) + (E), \quad i_T = i + Ei, \quad \frac{(E_T)}{(ES)} = \frac{n(E_T)}{n(ES)} = \frac{V_{max.}}{V},$$

$$K_s = \frac{(E)(S)}{(ES)} = \frac{n(E)(S)}{n(ES)}, \quad K_i = \frac{(E)(i)}{(Ei)} = \frac{n(E)((i_T) - (Ei))}{n(Ei)}$$

$$= \frac{n(E)(i_T)}{n(Ei)} - \frac{n(E)(Ei)}{n(Ei)} = \frac{n(E)(i_T)}{n(Ei)}$$

As (E) approaches zero $(n(E)(i_T))/(n(Ei))$ will approach K_i. Therefore $n(E)$ and $(n(E)(i_T))/(n(Ei))$ were plotted against each other, with use of the data of the experiment presented in Table III. A value of 0.6×10^{-9} M was obtained for K_i, from which n was calculated to be approximately 1.7. Thus the data suggested that only about 1/1.7 or 60 per cent of the FAD in this preparation was associated with active enzyme centers. The consistency of the calculated values for K_i, except with the lowest inhibitor concentrations, suggests the validity of the above presentation. This

approach gives an upper limit for the amount of enzyme present. There might be inactive enzyme present, still capable of combining with aldehyde; in addition, the aldehyde itself is slowly oxidized by the enzyme (see below), which means the amount of aldehyde present is overestimated.

TABLE III

Activity of Cream Enzyme toward Xanthopterin and AHP; Inhibition of This Activity by 6-Aldehyde

All values recorded as 10^{-9} mole per liter, except V which is moles per minute per mole of E_T. The symbols are defined in the text.

(S)	$\|6\text{-CHO }(i_T)\|$	$(E_T)^*$	(ES)	(E)	(Ei)	(i)	K_i
			$S = 2$-amino-4-hydroxypteridine†				
2590	0	9.8	51	8.0	1.8			
500	0	3.7	26.5	1.57	2.13			
128	0	3.7	10.2	0.60	3.10			
51	0	3.7	4.3	0.255	3.45			
2590	20.7	9.8	9.0	1.41	0.39	8.0	12.7	0.6
2590	5.5	9.8	24.8	3.89	1.08	4.8	0.7	0.2
51	5.5	3.7	0.90	0.053	0.73	2.92	2.6	0.6
51	2.4	3.7	2.08	0.123	1.74	1.84	0.56	0.5
51	1.1	3.7	2.98	0.177	2.50	0.92	0.18	0.5
			S = xanthopterin‡				
2650	0	9.8	17.6	9.2	0.6			
510	0	9.8	16.0	8.3	1.5			
132	0	3.7	7.6	1.50	2.20			
53	0	3.7	4.1	0.81	2.89			
2650	54.6	9.8	3.4	1.77	0.128	7.9	46.7	0.8
2650	20.7	9.8	6.0	3.12	0.225	6.5	14.2	0.5
53	5.5	3.7	0.77	0.15	0.55	3.0	2.5	0.5
53	2.4	3.7	2.03	0.40	1.45	1.85	0.55	0.4
53	1.1	3.7	2.90	0.57	2.07	1.06	0.04	(0.1)§

* Moles of enzyme = 0.6 × moles of FAD present.

† $V_{max.}$ = 62 moles per minute per mole of E_T. K_S = 0.72 × 10^{-6} mole per liter.

‡ $V_{max.}$ = 18.8 moles per minute per mole of E_T. K_S = 0.19 × 10^{-6}.

§ This value is less reliable than the rest due to the very low concentration of the aldehyde.

Other Substrates—It is of interest to compare the oxidation of AHP with that of other substrates attacked by the cream enzyme. Therefore, with the same enzyme preparation, the oxidation of xanthine, xanthopterin, and AHP was measured in 0.1 M phosphate buffer at pH 6.8. Since xanthine is not measurably fluorescent with present instruments, it was diffi-

cult to work with sufficiently low substrate concentrations to evaluate the Michaelis-Menten constant. However, by using absorption cells with a 10 cm. light path, it was possible to measure the oxidation with 1.2×10^{-6} M substrate, which gave a submaximal rate and therefore permitted calculation of the constant.

The different dissociation constants and maximal velocities are of interest (Tables III and IV). The observed values agree with the findings of Hofstee who estimated that the dissociation constants for xanthine and xanthopterin were of the order of 10^{-6} M, and that the xanthine constant was about 10 times larger than that of xanthopterin (11). If these oxidations are all effected by the same enzyme, then a single value should be obtained for the dissociation constant of the enzyme-inhibitor complex,

TABLE IV

Activity of Cream Enzyme toward Various Substrates; Inhibition by 6-Aldehyde

S is 10^{-6} mole per liter, V is moles per minute per mole of E_T (turnover number), and all the other values are 10^{-9} mole per liter.

Substrate	(S)	6-CHO (i_T)	(E_T)*	V	(ES)	(E)	(Ei)	(i)	K_i
AHP ($K_S = 340 \times 10^{-9}$)	37.0	0	86	48	85.2	0.79			
	37.0	250	86	11.5	20.6	0.19	65	185	0.54
Xanthopterin ($K_S = 100 \times 10^{-9}$)	43.0	0	86	12.8	85.8	0.20			
	43.0	250	86	6.6	44.2	0.10	42	208	0.51
Xanthine ($K_S = 900 \times 10^{-9}$)	43.0	0	86	234	84.2	1.8			
	43.0	250	86	37.6	13.5	0.27	72	178	0.67
	1.22	0	25						
6-Aldehyde ($K_S = 0.6 \times 10^{-9}$ (from K_i above))	35		1350	0.165					

* Assuming moles of enzyme $= 0.6 \times$ moles of FAD of enzyme.

K_i. Tables III and IV indicate that within experimental limits the same dissociation constant is obtained for all three substrates. This strongly suggests that one enzyme is responsible for all three oxidations. Hofstee has concluded that xanthine and xanthopterin are oxidized by the same enzyme (11).

Both Corran *et al.* (9) and Ball and Ramsdell (15) reported that milk or cream oxidase will oxidize reduced diphosphopyridine nucleotide (DPNH₂). This substrate[2] was oxidized by the present enzyme preparation with a molar velocity only 3 or 4 per cent as great as that with xanthine. The oxidation of DPNH₂ was unaffected by concentrations of 6-aldehydepteridine which completely blocked xanthine or AHP oxidation. It therefore

[2] The DPNH₂ was kindly supplied by Dr. F. Edmund Hunter.

seems likely that either two separate enzymes are involved or, as suggested by Corran *et al.* (9), that two separate active centers are present in the same enzyme.

The 6-aldehyde is itself slowly oxidized. If a small amount of the aldehyde is allowed to remain with the enzyme, its inhibitory capacity is slowly destroyed, in agreement with Kalckar *et al.* (10). A total of 70 μM of the
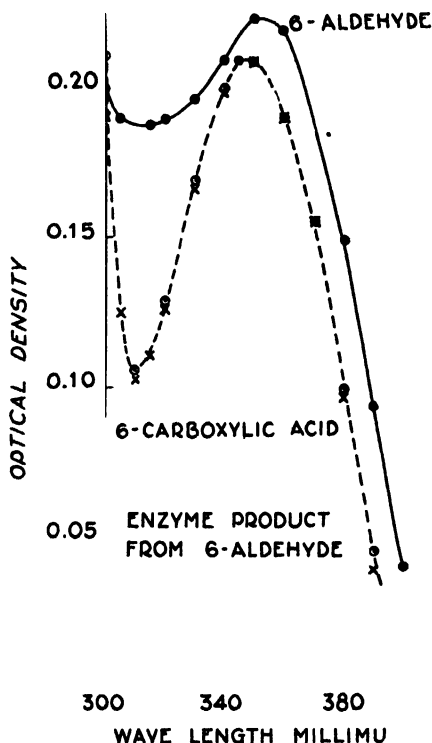

Fig. 2. The absorption spectra of 2-amino-4-hydroxy-6-formylpteridine, 2-amino-4-hydroxy-6-carboxypteridine, and the product obtained by treatment of the _aldehyde with cream enzyme.

6

6-aldehyde in 2 ml. of phosphate buffer of pH 6.8 was treated with an amount of the enzyme which contained 4.5 μM of FAD. The reaction was followed by measuring the decrease in optical density at 310 mμ. The reaction was complete in about 2 hours. The final optical absorption, after correction for the contribution of the enzyme, was identical with that of 2-amino-4-hydroxy-6-carboxylic acid (Fig. 2), proving that oxidation of the aldehyde group had occurred. The rate of oxidation of the aldehyde was a thousand times slower than that of xanthine (Table

IV). The turnover number was calculated to be 0.16; *i.e.*, an average of 6 minutes would be required for 1 molecule of enzyme to oxidize 1 molecule of this substrate. It was found that, when a molar excess of the 6-aldehyde was mixed with the enzyme for a few minutes and AHP (or other substrate) was then added, the initial velocity of AHP oxidation was almost zero but increased during a 2 or 3 minute period to a steady rate determined by the amount of inhibitor and substrate. On the other hand, when AHP and the 6-aldehyde were mixed together, and the enzyme was added last, the initial rate was not discernibly inhibited, but the velocity fell during the first 2 or 3 minutes to the same steady rate observed when the AHP was the last addition. The delay in the development of inhibition is presumably due to the slowness of the reaction enzyme + inhibitor → enzyme-inhibitor when they are both present in such high dilution. The delay in "deinhibition" resulting from the addition of the competing substrate AHP indicates that *both* the following reactions are slow: enzyme-inhibitor → enzyme + inhibitor, and enzyme-inhibitor → enzyme + oxidized inhibitor. Thus the turnover time of the last reaction must be several minutes at least in support of the directly observed rate of conversion of the 6-aldehyde to the 6-carboxyl compound. The opportunity presented for direct study of these reactions would seem to merit more thorough investigation.

<div align="center">DISCUSSION</div>

The existence of an inhibitor of purine oxidation, which is active in such low concentration as 10^{-9} M, may not be without biological consequences. If the 6-aldehyde were to be released slowly from PGA in the tissues, it might tend to preserve hypoxanthine or adenine from oxidation. PGA is known to reduce or eliminate the adenine requirement of certain microorganisms. Keith *et al.* have indeed shown that the xanthine oxidase activity of chick liver is increased 3-fold when the chicks are made deficient in PGA (16).

It is rather remarkable that the dissociation constants for xanthine, AHP, xanthopterin, and the 6-aldehyde (900, 340, 100, and 0.6×10^{-9} M) are roughly proportional to the calculated turnover numbers (238, 48, 13, and 0.16, respectively). This may be fortuitous; however, the apparent dissociation constants actually describe the situation,

$$E + S \underset{(2)}{\overset{(1)}{\rightleftharpoons}} ES \xrightarrow{(3)} E + \text{product}$$
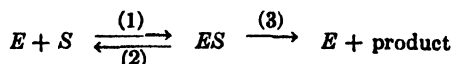
in which monomolecular reactions (2) and (3) are inseparable unless the reactions (1) are known (17). If reactions (2) are slow compared to reactions (3) (Case VI of Lineweaver and Burk (14)), then the apparent

dissociation constants would indeed parallel the velocity of oxidation. One consequence of the above parallelism is that in spite of a 1000-fold range of oxidation velocity between these four substrates, all four substances would be oxidized at about the same rate when present at concentrations of 10^{-9} M or less.

SUMMARY

1. 2-Amino-4-hydroxy-6-formylpteridine is a powerful inhibitor of the enzyme in cream which oxidizes xanthine, xanthopterin, and 2-amino-4-hydroxypteridine. The inhibitory constant appears to be the same relative to all three oxidations. It is approximately 0.6×10^{-9} M. Thus with low substrate and low enzyme concentrations, 10^{-4} γ per ml. will produce appreciable inhibition.

2. Because the amount of aldehyde inhibitor in actual combination with the enzyme is an appreciable fraction of the total added, it is possible to measure the combining proportions of enzyme and inhibitor and hence estimate the number of moles or equivalents of enzyme present. The amount of enzyme combining with 1 mole of aldehyde inhibitor contained not quite 2 moles of flavin-adenine dinucleotide. This might mean that about half of the flavin was inactive, or that 2 moles of flavin coenzyme were associated with each active center.

3. The inhibitor itself was slowly oxidized to 2-amino 4-hydroxy-6-carboxylic acid. The turnover number for this reaction was about 0.16, compared to turnover numbers of 234, 48, and 13 observed for xanthine, 2-amino-4-hydroxypteridine, and xanthopterin, respectively, as substrates. The slow turnover number for the aldehyde was confirmed by the lag period of several minutes for "deinhibition" when substrate was added to the enzyme after the inhibitor.

4. The oxidation of reduced diphosphopyridine nucleotide by the enzyme from cream was not inhibited by the 6-aldehyde, and hence another active group or another enzyme is probably responsible for its oxidation.

BIBLIOGRAPHY

1. Lowry, O. H., Bessey, O. A., and Crawford, E. J., *J. Biol. Chem.*, **180**, 389 (1949).
2. Kalckar, H. M., *J. Biol. Chem.*, **167**, 429 (1947).
3. Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, **172**, 349 (1948).
4. Lowry, O. H., *J. Biol. Chem.*, **173**, 677 (1948).
5. Bessey, O. A., Lowry, O. H., and Love, R. H., *J. Biol. Chem.*, in press.
6. Kornberg, A., *J. Biol. Chem.*, **174**, 1051 (1948).
7. Ball, E. G., *J. Biol. Chem.*, **128**, 51 (1939).
8. Kalckar, H. M., *J. Biol. Chem.*, **167**, 461 (1947).
9. Corran, H. S., Dewan, J. G., Gordon, A. H., and Green, D. E., *Biochem. J.*, **33**, 1694 (1939).

10. Kalckar, H. M., Kjeldgaard, N. O., and Klenow, H., *J. Biol. Chem.*, **174,** 771 (1948).
11. Hofstee, B. H. J., *J. Biol. Chem.*, **179,** 633 (1949).
12. Philpot, J. S. L., *Biochem. J.*, **33,** 1707 (1939).
13. Michaelis, L., and Menten, M. L., *Biochem. Z.*, **49,** 333 (1913).
14. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658 (1934).
15. Ball, E. G., and Ramsdell, P. A., *J. Biol. Chem.*, **131,** 767 (1939).
16. Keith, C. K., Broach, W. J., Warren, D., Day, P. L., and Totter, J. R., *J. Biol. Chem.*, **176,** 1095 (1948).
17. Briggs, G. E., and Haldane, J. B. S., *Biochem. J.*, **19,** 338 (1925).

PURIFICATION AND PROPERTIES OF DEHYDROPEPTIDASES*

By JOSEPH SHACK†

(*From the National Cancer Institute, National Institutes of Health, Bethesda, Maryland*)

The discovery of an enzyme system that catalyzed the hydrolysis of dehydropeptides to ammonia and the corresponding amino and keto acids was reported in 1932 by Bergmann and Schleich (2). Greenstein and Leuthardt (3) have studied the distribution of dehydropeptidase activity in a variety of tissues, and from the finding that all of the tissues acted on glycyldehydroalanine (GdA) but only a few on chloroacetylde-hydroalanine (CdA), they postulated that there were at least two dehydropeptidases. The enzyme that hydrolyzed GdA was designated dehydropeptidase I (DHP I) and the one that hydrolyzed CdA was called dehydropeptidase II (DHP II).

There are reported in this paper methods for separation and purification of DHP I and DHP II from beef kidney, and studies of their specificity and kinetic properties. Certain data bearing on the relation of these enzymes to those concerned with hydrolysis of the analogous saturated peptides are also presented.[1]

EXPERIMENTAL

Assay of Dehydropeptidase Activity—Dehydropeptidase activity was assayed by spectrophotometric methods similar to that described by Carter and Greenstein (4). GdA was used as substrate for DHP I and CdA for DHP II in following the purifications. In the present study the reaction mixtures for routine assay of activity contained appropriate amounts of enzyme, and were 0.01 M in substrate and 0.04 M in borate buffer at pH 8.3. They were incubated at 37°; aliquots were removed at intervals and diluted twenty times with distilled water. The optical density of each sample was immediately measured at 250 mμ by means of a Beckman spectrophotometer against the corresponding comparison blank. Under these conditions (with both crude extracts and purified preparations) the change of optical density with time is linear up to about

---

50 per cent hydrolysis, and the initial rates are proportional to enzyme concentration at rates up to about 50 per cent hydrolysis per $\frac{1}{2}$ hour.

1 enzyme unit is here defined as the amount of enzyme causing hydrolysis of 1 μM of substrate per hour, and the specific activity A is given in micromoles per hour per mg. of N by the ratio, units per mg. of enzyme N. Specific activities were calculated from the initial linear portions of time curves. The specific activity with respect to a given substrate is designated by a subscript (for example A_{GdA} or A_{CdA}).

Separation by Differential Centrifugation—Carter and Greenstein (4) have reported a 6-fold purification of DHP I from rabbit kidney, and Yudkin and Fruton (5) a several fold purification from rat kidney of the enzyme attacking glycyldehydrophenylalanine. In the present study attempts to purify DHP I by ethanol or salt fractionation yielded a product at most 5 to 7 times as active as the original extract, but the behavior of the material suggested that the enzyme might be associated with particulate material. This was confirmed by differential centrifugation of fresh beef kidney homogenates. The pellet that formed on centrifugation at 2000 $\times$ g for 15 minutes had only slight activity. The pellet that formed on centrifugation at 20,000 $\times$ g for 2 hours contained approximately 85 per cent of the DHP I activity of the original homogenate; practically all of the DHP II was found in the last supernatant. Essentially identical results were found with rat kidney. It was immaterial whether the homogenates were prepared by grinding with sand or with the Waring blendor. By contrast 80 to 90 per cent of the DHP I of beef and rat liver and of primary rat hepatoma was found to remain in the last supernatant on differential centrifugation. These findings are paralleled by the solubility of the activities in ethanol-water mixtures at $-5°$ when 0.3 volume of 95 per cent ethanol was added to each homogenate and the mixtures were filtered. In the case of liver and rat hepatoma, about 85 per cent of the DHP I activity of the homogenate is found in the clear filtrate, but with kidney only 1 to 3 per cent is recovered in the filtrate. In every case DHP II was found to be soluble in the ethanol-water mixture.

These results indicate a difference in the state of aggregation of DHP I among various tissues. That of kidney is apparently associated with what Claude (6) has called the microsome fraction. A number of procedures, including prolonged extraction, autolysis under toluene, freezing and thawing, and digestion by proteolytic enzymes, have been tried without success in attempts to free the activity from the particulate material. However, a 25-fold increase in specific activity was carried out by a procedure that combines differential centrifugation with partial digestion of the particulate material by trypsin. A typical preparation follows.

Purification of Dehydropeptidase I—Fresh beef kidney was freed of fat, ground several times in a meat grinder, and stirred mechanically for 1 hour at room temperature with 4 volumes of distilled water. The mixture was strained through cheese-cloth and centrifuged for 15 minutes at 3000 $\times$ g. The turbid supernatant (3.5 mg. of N per ml., $A_{GdA} = 269$) was centrifuged for 1 hour at 15,000 R.P.M. in the Sharples centrifuge, and the precipitate was resuspended in 0.05 M $NaHCO_3$ (pH of suspension 8.2). To 160 ml. of the suspension (0.64 mg. of N per ml., $A_{GdA} = 507$; 51,900 total units of DHP I) was added about 0.5 mg. of crystalline trypsin. The amount of trypsin in the aliquots of the preparation that were used for assay was found to have no measurable action on any of the sub- strates studied in this investigation. On standing at room temperature a visible clarification of the suspension occurred; after 6 hours it was di- alyzed for 12 hours against cold distilled water. The preparation now contained 59,000 units of DHP I (0.37 mg. of N per ml., $A_{GdA} = 992$). Solid $NaHCO_3$ to pH 7.8 and 0.5 mg. of trypsin were added; after stand- ing for 6 hours the mixture was dialyzed as before for 12 hours. The suspension, which contained 98,600 units of DHP I (0.24 mg. of N per ml., $A_{GdA} = 2560$), was now centrifuged at 20,000 $\times$ g for 2 hours at 2°. The pellet was suspended in 35 ml. of distilled water. This suspension contained 69,000 units of DHP I and 0.34 mg. of N per ml.; its A_{GdA} was 5750. It was light tan in color and somewhat turbid. When the suspen- sion was stored without preservative in the refrigerator, the activity was stable for months. No further increase in activity could be achieved by repetition of the above procedures.

In several instances a considerable increase in total units of DHP I activity as well as in specific activity resulted from the action of trypsin on the particulate matter. Whether this is due to an activation, to de- struction of an inhibitor, or to increase of surface area is not yet clear.

Purification of Dehydropeptidase II—The hydrolysis of GdA by beef kidney extracts is about 200 times as rapid as that of CdA, A_{GdA} ranging from about 175 to 300, A_{CdA} from 0.8 to 2.0. However, preparations of DHP II, which are over 1000 times as active per mg. of N as the original extract toward CdA and almost free of action on GdA, have been made by ethanol fractionation at low temperatures. The procedure follows; ethanol was added as 60 per cent ethanol, and the temperature at each stage was kept close to the freezing point of the solution.

Beef kidney is freed of fat, ground several times in a meat grinder, stirred mechanically for 1 hour with several volumes of distilled water, and strained through gauze. The ethanol concentration is brought to 17 per cent, and after 12 hours the precipitate is collected in the Sharples centrifuge and discarded. The supernatant contains about 24 per cent of the protein, only 1 per cent of the DHP I activity, but practically all

of the DHP II activity of the original extract. The pH of the solution is brought to 5.6 with N acetic acid and after 12 hours the bulky precipitate is removed in the Sharples centrifuge.[2] The supernatant is now clarified by addition of Celite and filtration. The pH of the solution is brought to 4.9 with N acetic acid, and after 24 hours the slight precipitate is collected in the Sharples centrifuge. This precipitate contains about 60 per cent of the total units of DHP II of the original extract; in several different preparations the A_{CdA} at this stage ranged between 72 and 116.

The above precipitate is suspended in water (protein concentration about 0.2 per cent) and the pH is adjusted to 7.0 with 0.1 N NaOH. After stirring for 1 hour insoluble material is removed by filtration. The ethanol concentration is brought to 17 per cent, and the pH to 5.5 with 0.1 N acetic acid; after 12 hours the precipitate is removed in the centrifuge. The pH of the supernatant is now adjusted to 4.9 with 0.1 N acetic acid. The active precipitate is collected in the centrifuge after 24 hours and again treated by the procedure of this paragraph. Three or four such precipitations yield a product with a specific activity close to 1000, but no significant increase in purity has been achieved by further repetition. Recovery at this stage is about 15 per cent of the activity of the original extract. Such a preparation has been used for many of the kinetic studies described below.

For further purification a 0.5 per cent solution of material with an A_{CdA} of around 1000 is dialyzed against 0.02 M acetate buffer of pH 4.9 for 12 hours. A small amount of flocculent precipitate is removed by centrifugation. On standing an increasing turbidity develops, and microscopic examination has shown the presence of what appear to be very minute plates. After 2 days the active precipitate is collected by centrifugation. The most active material made by this procedure (DHP II, Preparation X) had a specific activity of 2540, and neither repetition of the dialysis nor any other procedure tried so far has increased this value. This preparation was found to be homogeneous in the ultracentrifuge. It is estimated on the basis of a 1000-fold purification that beef kidney contains approximately 30 mg. of DHP II per kilo of fresh tissue.

Kinetic Properties—In Fig. 1 are shown the pH-activity curves of DHP I and DHP II. The sharp optimum at pH 8.3 of DHP I is in contrast to the broad zone of activity of DHP II. Yudkin and Fruton (5) have reported that the pH optimum for the action of rat kidney dehydropeptidase on glycyldehydrophenylalanine is at pH 8.0. The author, in agreement with Yudkin and Fruton (5), did not find the marked inhibi-

[2] The pH values of the 17 per cent ethanol solutions refer to those measured at room temperature on aliquots diluted with distilled water to ethanol concentrations of 5 per cent.

tion of dehydropeptidase activity by phosphate that was reported by Carter and Greenstein (4).

Fig. 2 shows the dependence of rate on concentration of substrate. The Michaelis-Menten constants are estimated to be 1.4×10^{-3} M for DHP I and 2.5×10^{-3} M for DHP II.

At low substrate concentrations the action of DHP I on GdA and of DHP II on CdA closely followed the kinetics of first order reactions to practically complete hydrolysis. This is shown in Table I. The
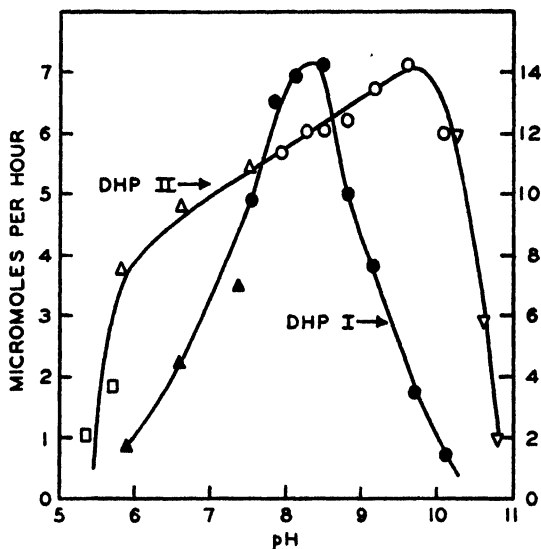

FIG. 1. pH-activity curves of dehydropeptidases I and II. Ordinate values on left for DHP I per 0.0012 mg. of enzyme nitrogen; values on right for DHP II per 0.01 mg. of enzyme nitrogen. O and ●, borate; ▽, carbonate; △ and ▲, phosphate; □, acetate buffers; all 0.04 M; temperature 37°.

kinetics of hydrolysis of these aliphatic substrates contrast with those reported for glycyldehydrophenylalanine by Yudkin and Fruton (5), who found that the initial rate followed the kinetics of a first order reaction, but that the first order velocity constants decreased after about 35 per cent hydrolysis, and that the rate was negligibly slow after 65 per cent hydrolysis. We have observed results similar to theirs with DHP I and glycyldehydrophenylalanine. An explanation for the differences may be found in our earlier report (7) that the hydrolysis of glycyldehydrophenylalanine occurs in two steps that can be differentiated by the spectrophotometric method, the first step being enzymatic, the second involving the spontaneous breakdown of an intermediate.

FIG. 2. Dependence of action of dehydropeptidases I and II on substrate concentration. Ordinates, initial rates in micromoles of substrate hydrolyzed per hour per mg. of enzyme nitrogen; values on left for DHP I and GdA; values on right for DHP II and CdA. Abscissa, initial concentration of substrate in micromoles per ml. of reaction mixture. Temperature 37°; pH 8.3; 0.04 M borate buffers.

TABLE I

Kinetics of Action of Dehydropeptidases I and II at Low Substrate Concentrations

System	t	Hydrolysis	$k \times 10^2$	System	t	Hydrolysis	$k \times 10^2$
	min.	*per cent*			*min.*	*per cent*	
GdA 0.0005 M, 0.00072 mg. DHP I nitrogen per ml.; $D_1 = 1.48$, $D_2 = 0.14$	5	10.5	1.05	CdA 0.0005 M, 0.007 mg. DHP II nitrogen per ml.; $D_1 = 1.33$, $D_2 = 0.10$	10	25.6	1.27
	10	20.2	0.98		20	44.0	1.27
	20	36.1	0.97		30	60.0	1.33
	30	49.3	0.98		40	69.5	1.27
	40	59.1	0.97		60	85.5	1.37
	60	74.5	0.99		90	95.2	1.45
	90	87.0	0.98				
	135	94.5	0.93				
	180	97.4	0.88				

D_1 = optical density of the solution at $t = 0$; D_2 = optical density after complete hydrolysis. Solutions at pH 8.3, 0.02 M borate buffers. Temperature 37°.

k = first order reaction constant according to $k = 1/t \log (100/(100 - \text{per cent hydrolysis}))$.

Action of Inhibitors—Bergmann and Schleich (2) found that dehydropeptidase was inhibited by cyanide. Yudkin and Fruton (5) have re-

ported that hydrolysis of glycyldehydrophenylalanine by a rat kidney preparation was inhibited by cyanide, cysteine, and hydrogen sulfide.

The effect of various inhibitors on our preparations of DHP I and DHP II has been tested. Neither was inhibited by 0.02 M sodium azide, 0.02 M potassium iodide, or 0.02 M sodium fluoride. Both are completely inhibited by 0.002 M sodium cyanide and by 0.02 M sodium thioglycolate. However, 0.02 M sodium iodoacetate completely inhibits DHP II but is without effect on DHP I.

Preliminary studies on the relation of the degree of inhibition by cyanide or iodoacetate to the concentration of the inhibitor resulted in first order sigmoidal curves suggestive of reversible equilibria.[3] The reversibility of these inhibitions has been shown by dialysis experiments. The inhibitors were added to solutions of the enzymes to concentrations of 0.1 M and the solutions were then dialyzed against distilled water. The inhibition of both enzymes by cyanide and by thioglycolate and of DHP II by iodoacetate proved to be reversible, since the original activities were restored to within a few per cent on removal of the inhibitors by dialysis.

It may be that these inhibitions are due to involvement of a metal in dehydropeptidase activity. Such an involvement has been suggested by Yudkin and Fruton (5) and they have reported (8) that their preparation was partially inactivated by long dialysis against demineralized water, the activity being restored by addition of zinc chloride. With our preparations, neither long dialysis against distilled water nor against 0.01 M cyanide followed by distilled water led to any significant decrease of activity; however, no special precautions had been taken to free the distilled water of traces of metals.

Studies of competitive inhibition have also shown that the action of DHP I on GdA is not measurably inhibited by CdA, nor that of DHP II on CdA by GdA.

Specificity of Dehydropeptidases

Action on Dehydropeptides—The action of typical preparations of DHP I and DHP II on a series of dehydropeptides, all with a free terminal carboxyl group, is summarized in Table II.

DHP I acts on only three of the compounds, all of which have a free amino group α to the susceptible bond. The specific activities with respect to these three substrates are all about 25 times those of crude beef kidney extract. However, because of the association of the activities with particulate material, it cannot be assumed that the three substrates are hydrolyzed by a single enzyme, even though no appreciable separation has been made by the purification procedures.

[3] Unpublished data of the author.

It is seen from Table II that DHP II rapidly hydrolyzes acetyldehydroalanine as well as CdA. Inasmuch as the ratio of these activities did not change significantly during the purification and was constant for several preparations, it is likely that both substrates are attacked by the same enzyme. This is also indicated by a mixed substrate experiment in which the hydrolysis of a mixture of CdA and acetyldehydroalanine (both 0.01 M) by DHP II was measured both spectrophotometrically and by production of ammonia. The over-all rate was not the sum, but close to an average, of the separate rates.

TABLE II

Action of Dehydropeptidases I and II on Dehydropeptides

Substrate	Specific activity*	
	Dehydropeptidase I	Dehydropeptidase II
Glycyldehydroalanine	5750	180
Glycyldehydrophenylalanine	2610	0
DL-Alanyldehydroalanine	3900†	138†
Chloroacetyldehydroalanine	17	2380
Chloroacetyldehydrophenylalanine	0	0
DL-α-Chloropropionyldehydroalanine	0	0
Acetyldehydroalanine	0	1560
Acetyldehydrophenylalanine	0	0
Acetyldehydroleucine	0	0
Acetyldehydrotyrosine	0	0

* Specific activities in micromoles of substrate hydrolyzed per hour per mg. of enzyme N in 0.04 M borate buffer at pH 8.3 and 37°. Substrates 0.01 M (racemic substrates 0.02 M).

† Only one optical component hydrolyzed.

In addition, DHP II possessed a small but definite capacity to hydrolyze GdA and DL-alanyldehydroalanine. Whether these low residual activities are due to a slight contamination by DHP I or to a degree of cross-specificity remains an open question at present. However, were they due to contamination by DHP I, a measurable action on glycyldehydro-phenylalanine would be expected unless DHP I were multiple and the purification procedures had completely eliminated from DHP II only that component specific for glycyldehydrophenylalanine.

Action on Analogous Saturated Peptides—In view of the rapid hydrolysis by tissue extracts of glycyl-DL-alanine (GA) (9, 10) and chloroacetyl-DL-alanine (CA) (11), it was considered of interest to determine whether preparations of DHP I and DHP II attacked these peptides. Dr. Price had assayed two of the partially purified preparations and found that

the DHP I had some action on GA, and that the DHP II hydrolyzed CA far more rapidly than it hydrolyzed CdA.[4] We have assayed some of the more highly purified preparations and found similar results.

The hydrolysis of GA and CA was assayed by measuring the increase of carboxyl groups by the Linderstrøm-Lang acetone titration. The reaction mixtures were 0.125 M in substrate and 0.05 M in borate buffer at pH 8.3. Specific activities were calculated from initial portions of time curves as micromoles of substrate hydrolyzed per hour per mg. of enzyme N. Typical results at 37° are presented in Table III, which also includes data for crude beef kidney extracts as well as the values of A_{GdA} and A_{CdA} for the same preparations.

TABLE III

Action of Dehydropeptidases I and II on Analogous Dehydropeptides and Peptides

Substrate	Specific activity*		
	Crude beef kidney extract	Dehydro-peptidase I	Dehydro-peptidase II
Glycyldehydroalanine	230	4200	180
Glycyl-DL-alanine	5300†	3260†	25,000‡
Chloroacetyldehydroalanine	1.5	15	2,380
Chloroacetyl-DL-alanine	520‡	75‡	208,000‡

* All specific activities in micromoles of substrate hydrolyzed per hour per mg. of enzyme N under conditions described in the text.
† Both optical components hydrolyzed.
‡ Only one optical component hydrolyzed.

Table III shows that A_{GA} and A_{CA} of purified DHP I are both less than the corresponding values of the crude tissue, whereas A_{GdA} is increased 20-fold.

In the case of DHP II, A_{GA} is about 5 times that of the crude extract, but both A_{CA} and A_{CdA} are at least several hundred times as high as in the original tissue. The action of several different preparations of DHP II of varying degrees of purity on CA and CdA has been assayed; in every case the ratio $A_{CA}:A_{CdA}$ was found to be close to 90 (± 3). Typical pairs of values of A_{CA} and A_{CdA} respectively for five different preparations were 21,160 and 245; 33,000 and 354; 95,500 and 1065; 208,000 and 2380; 230,000 and 2540. In addition, the ratio remained essentially constant during a given isolation from the first precipitation at pH 4.9 through the final step. Such a correspondence raises the question of whether these two substrates are hydrolyzed by a single enzyme that has a multiple specificity, or by two separate enzymes that have very closely similar physicochemical prop-

[4] Price, V. E., personal communication.

erties. Further information on this point was sought through sedimentation and electrophoresis of DHP II preparations.

Ultracentrifugal Analysis of DHP II — A solution of DHP II (Preparation X, A_{CdA} = 2540, A_{CA} = 230,000) was examined in the Spinco[5] electrically driven ultracentrifuge.[6] As is shown in Fig. 3, the material sedimented as a single component with a sedimentation constant (S_{20w}) of 4.9. In the absence of diffusion data the exact molecular weight cannot be calculated, but it is estimated to approximate that of serum albumin (12). With a value of 70,000, the turnover numbers of the preparation have been calcu-
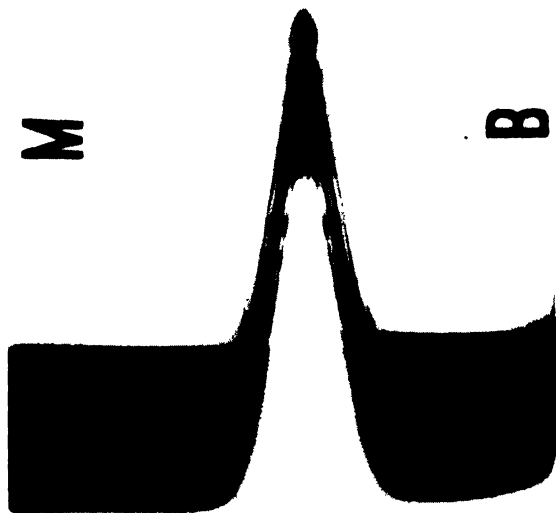
Fig. 3. Sedimentation pattern of DHP II in acetate buffer. pH 5.6; ionic strength 0.05 in sodium acetate, 0 10 in NaCl. Protein concentration 0.70 per cent. Speed 59,900 R.P.M. Photograph taken 80 minutes after reaching full speed. M, meniscus; B, bottom of cell.

lated as 495 for CdA and 45,000 for CA (in moles of substrate hydrolyzed per mole of enzyme per minute at 37°).

Electrophoresis of DHP II — It was not possible to examine electrophoretically a sample of DHP II of the highest purity, owing to the small amount of such material available. Consequently, a preparation with an A_{CdA} of 1065 and an A_{CA} of 95,500 was used in an electrophoretic sampling experiment; a 0.75 per cent solution of this material in borate buffer at pH 8.68 (ionic strength 0.02 in sodium borate, 0.08 in NaCl) was examined. The

[5] Specialized Instruments Corporation, Belmont, California.

[6] The author is indebted to Dr. Gerson Kegeles for carrying out the electrophoretic and ultracentrifugal runs.

isoelectric point of DHP II is estimated from minimum solubility to be around 4.9. The electrophoretic pattern showed a single sharp peak corresponding to a mobility at 1° of -5.84 cm.^{2} sec.$^{-1}$ volt^{-1}, and also a small amount of faster moving components that were estimated to comprise less than 5 per cent of the total protein. After 133 minutes of electrolysis (potential gradient 4.25 volts per cm.), 2 ml. of solution were removed by a capillary from the center of the main peak on the descending side. This aliquot had a protein concentration of 0.068 per cent; its A_{CdA} was 1000 and its A_{CA} 100,500. The close agreement of these values with those of the solution before electrophoresis indicates that the activities of DHP II toward CdA and CA are associated with the same electrophoretic component.

DISCUSSION

It is evident from Table II that DHP I and DHP II are distinct enzymes with quite different specificities. In addition, differences in the state of aggregation, kinetic properties, pH optima, and inhibition were also found.

The parallelism between the capacity of preparations of DHP II to hydrolyze CdA and CA, as well as evidence from sedimentation and electrophoresis, has raised the question of whether these two substrates are hydrolyzed by a single enzyme that has a multiple specificity. Other examples of what appears to be multiple specificity have been reported for xanthine oxidase (13), chymotrypsin (14), L-amino acid oxidase (15), and trypsin (16). Studies on DHP II are not yet sufficiently extensive to determine conclusively whether we are in this case dealing with a single enzyme or with a mixture of two enzymes that have almost identical physicochemical properties. In any case, the method described for purification of the activity toward CdA also achieves a several hundred fold purification of the activity toward CA, and, should the preparation prove to be a mixture, it will serve as the source of a very highly active peptidase as well as of a dehydropeptidase if methods of separation are found. This problem is being investigated, and it is expected that additional kinetic studies as well as attempts at further fractionation will provide more evidence on the question.

SUMMARY

1. Two distinct dehydropeptidases have been isolated from beef kidney. Dehydropeptidase I was found to be associated with particulate material, and a 25-fold purification was carried out by a method that combined differential centrifugation and partial digestion of the particles by trypsin. A method involving ethanol fractionation at low temperatures has been described by which preparations of dehydropeptidase II with specific

activities over 1000 times that of the crude tissue extract have been made. The purest dehydropeptidase II was homogeneous with respect to sedimentation in the ultracentrifuge.

2. Dehydropeptidase I acts on glycyldehydroalanine and certain other dehydropeptides that have an α-amino group. Dehydropeptidase II is specific toward acetyl- and chloroacetyldehydroalanine.

3. Various kinetic properties of the two enzymes have been compared. Dehydropeptidase I has a sharp optimum of activity at pH 8.3. Dehydropeptidase II exhibits a broad zone of activity with a maximum at pH 9.7. Both enzymes are inhibited by cyanide and by thioglycolate, and dehydropeptidase II by iodoacetate as well. These inhibitions are completely reversed on removal of the inhibitors by dialysis.

4. Data on the action of the dehydropeptidase preparations on glycyl-DL-alanine and chloroacetyl-DL-alanine are presented. A close correspondence was found between the capacity of dehydropeptidase II to hydrolyze chloroacetyldehydroalanine and chloroacetyl-DL-alanine, and the question of whether these two compounds may be hydrolyzed by a single enzyme with a mutiple specificity was discussed.

BIBLIOGRAPHY

1. Shack, J., *Cancer Res.*, **7**, 713 (1947).
2. Bergmann, M., and Schleich, H., *Z. physiol. Chem.*, **205**, 65 (1932).
3. Greenstein, J. P., and Leuthardt, F. M., *J. Nat. Cancer Inst.*, **6**, 197 (1946).
4. Carter, C. E., and Greenstein, J. P., *J. Nat. Cancer Inst.*, **7**, 51 (1946).
5. Yudkin, W. H., and Fruton, J. S., *J. Biol. Chem.*, **169**, 521 (1947).
6. Claude, A., *J. Exp. Med.*, **84**, 253 (1946).
7. Shack, J., *Arch. Biochem.*, **17**, 199 (1948).
8. Yudkin, W. H., and Fruton, J. S., *J. Biol. Chem* , **170**, 421 (1947).
9. Maschmann, E., *Biochem. Z.*, **313**, 129 (1943).
10. Merton, R., *Biochem. Z.*, **318**, 167 (1947).
11. Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **175**, 969 (1948).
12. Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford, 372 (1940).
13. Ball, E. G., *J. Biol. Chem.*, **128**, 51 (1939).
14. Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **145**, 253 (1942).
15. Blanchard, M., Green, D. E., Nocito, V., and Ratner, S., *J. Biol. Chem.*, **161**, 583 (1945).
16. Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J. E., *J. Biol. Chem.*, **172**, 221 (1948).

MUTANT STRAINS OF ESCHERICHIA COLI UNABLE TO SYNTHESIZE p-AMINOBENZOIC ACID

By J. O. LAMPEN,* M. J. JONES, AND R. R. ROEPKE

(From the Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, Stamford, Connecticut)

Among the growth factor-requiring strains of *Escherichia coli* obtained in this Laboratory by x-ray treatment or prolonged serial transfer (1, 2) were three strains which grew when p-aminobenzoic acid (PABA) was added to the basal medium. The importance of PABA in the metabolism of microorganisms was first indicated when Woods (3) reported that the bacteriostatic action of the sulfonamides is competitively antagonized by the addition of PABA. Rubbo *et al.* (4) soon found *Clostridium aceto-butylicum* requires the addition of PABA for growth, and a number of other organisms were subsequently reported to require PABA (see Peterson and Peterson (5) for references). Mutant strains which require PABA were obtained after irradiation with x-rays of the fungi *Neurospora* (6) and *Ophiostoma* (7). In addition, PABA is a part of the pteroylglutamic acid (folic acid) molecule (8), and the synthesis of pteroylglutamic acid from PABA by certain enterococci and lactobacilli has been reported (9, 10) to be inhibited by sulfonamides. It was therefore of interest to study the growth requirements of these mutants and especially to investigate the activity of various compounds related to PABA.

Most of the experiments reported in this communication were performed with mutant 273-384. Some data obtained with mutants 1861-460 and 45-462 are presented as well. A preliminary report of a portion of this work has previously been published (11).

Procedures

The mutant strains were produced and isolated by the general methods previously employed (1, 2). Mutant 273-384 was isolated from a single colony culture of *E. coli* irradiated at 40 kv. and 20 ma. by use of a copper target for 4 hours during growth in Difco A. C. broth at 30°.[1] Mutants 1861-460 and 45-462 were isolated from resting cell suspensions irradiated at 40 kv. and 20 ma. for 2 hours at 12–15°.

---

* Present address, Department of Microbiology, School of Medicine, Western Reserve University, Cleveland, Ohio.
[1] Mutant 273-384 can be obtained from the American Type Culture Collection as No. 9723a, and the parent strain of *E. coli* as No. 9723.

The general bacteriological techniques employed have been described previously (12). The basal, synthetic medium was that of MacLeod (13) with the following composition (per liter): NaCl 5.0 gm., $(NH_4)_2SO_4$ 4.72 gm., KH_2PO_4 2.72 gm., glucose 2.0 gm., asparagine 2.0 gm., and 1.7 mg. each of $FeCl_2$, $MgCl_2$, and $CaCl_2$. The medium was adjusted to pH 7.0 to 7.1 with NaOH. Unless otherwise stated all supplements were sterilized by autoclaving with the basal medium.

The amino acid mixture used for supplementation of the basal medium was essentially that of Hac, Snell, and Williams (14). However, the DL forms of leucine, lysine, and aspartic acid were used instead of the natural materials. A stock solution was prepared containing, per ml., 8 mg. of DL-aspartic acid, 2 mg. each of the other DL acids, 10 mg. of L-glutamic acid, and 1 mg. each of the other L acids. This solution, as prepared from commercial amino acids, contained traces of PABA. This was removed by adsorption at pH 3.5 with 1 gm. of Darco G-60 per 100 ml. The purified material is subsequently termed amino acid supplement. The purine supplement consists of 5 γ each of adenine, guanine, and xanthine per ml. of final medium.

Results

Response of Mutant 273-384 to PABA under Various Conditions—The addition of PABA to the synthetic basal medium is sufficient to support good growth of the mutant strain. In the presence of excess PABA (0.1 γ per ml.) the turbidity attained by the mutant at 16 hours of incubation equals that attained by the parent strain of *E. coli*. However, the response of mutant 273-384 to limiting concentrations of PABA is increased very markedly by the addition of a mixture of amino acids and purines to the basal medium (Table I). In the absence of the amino acids the addition of the individual purines delayed growth, although with either guanine or xanthine the final turbidity was greater. In the presence of the amino acids any one of the purines stimulated growth. Uracil and cytosine were inactive. The stimulation observed with the entire amino acid supplement could often be duplicated by the addition of DL-methionine alone. This compound also prevented the growth inhibition by the purines. However, the growth of the mutant has generally been better when the entire mixture was present and this has customarily been used. Also 5 γ per ml. each of adenine, guanine, and xanthine were generally added instead of a single purine. The concentration of PABA at which half maximal growth occurred at 16 hours was 0.0012 γ per ml. on the unsupplemented medium. This requirement was reduced to 0.0005 γ per ml. by the addition of the amino acid supplement and to 0.0001 γ by the further addition of the purine supplement. The rate of growth and final

turbidity reached by the mutant in the presence of the amino acid and purine supplements and excess PABA (0.1 γ per ml.) were approximately the same as those obtained under identical conditions with the parent strain of *E. coli*.

Wyss, Lilly, and Leonian (15) reported that the concentration of added PABA required for the growth of an *aminobenzoicless* mutant of *Neurospora crassa* decreased as the pH of the medium decreased. They concluded that this organism was permeable only to the un-ionized form of the acid. We

TABLE I

Effect of Amino Acids and Purines on Response of Mutant 273-384 to p-Aminobenzoic Acid

Compound	Additions per ml.	Additions to basal medium				
		None	0.001 γ PABA per ml.		Amino acid supplement* + 0.00008 γ PABA per ml.	
		Turbidity readings† (average of duplicate tubes)				
		16 hrs.	16 hrs.	64 hrs.	16 hrs.	64 hrs.
None........		0	21	26	5	5
Adenine (A).............	10 γ	0	0	19	23	33
Guanine (G)...	10 "	0	11	37	15	23
Xanthine (X)..	10 "	0	12	37	27	39
Hypoxanthine. . . .	10 "	0	0	22	18	28
Uracil... . . .	10 "	0	22	26	5	5
Cytosine	10 "	0	24	23	4	6
A + G + X.	5 " (Each)	0	10	32	28	35
DL-Methionine.	10 "	0	38	51	5	5
Amino acid supplement .. .	0.02 ml.	4	39	43		
" " " ..	0.02 "					
+ A + G + X...............	5 γ (Each)	6	74	72		

* 0.02 ml. per ml.

† 1 unit is equivalent to 13.4 million cells of *E. coli* per ml. (*cf.* (12)).

tested the effect of the acidity of the medium on the response of mutant 273-384 to PABA. In experiments with the unsupplemented basal medium the concentration of PABA required did not change markedly over the range of pH 5.2 to 7.2; however, growth was slower in the more acid media. The results obtained in the presence of the amino acid and purine supplements are illustrated in Fig. 1. Citrate buffers were used in the experiments which are shown here. The response curve at pH 6.4 was essentially unchanged when M/30 phosphate buffer was substituted for the citrate. The results with phosphate buffers at pH 6.9 and 7.2 (pH 7.0 and 7.5 before autoclaving) were similar to those at pH 6.4, although the tur-

bidity reached with excess PABA was somewhat greater in the more alkaline media. It can be seen from Fig. 1 that the sigmoid curve obtained at pH 6.4 and above was replaced by a more nearly linear curve at pH 5.7 and 6.1. However, the concentration of PABA required for half maximal growth remained approximately the same (about 0.0001 γ per ml.). At pH 5.2 about 4 times as much PABA were required. These data offer no
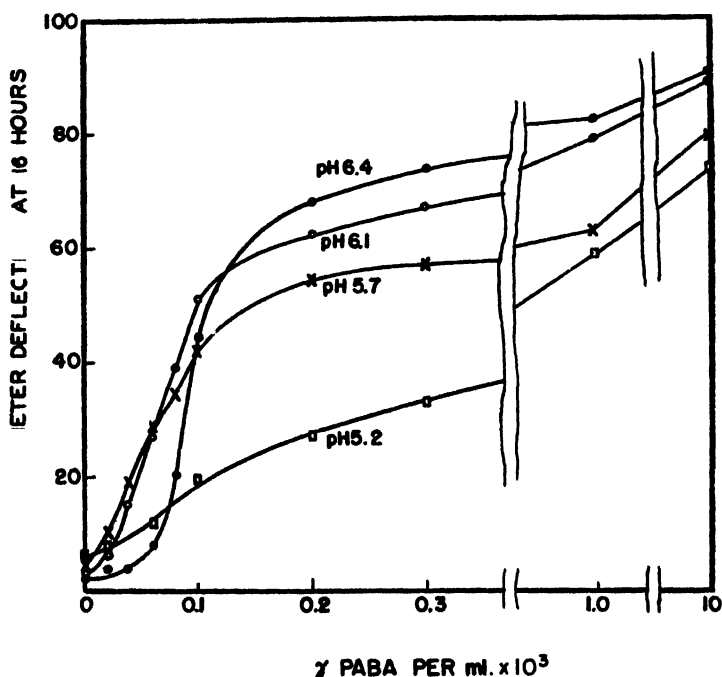
FIG. 1. The effect of the pH of the medium on the response of mutant 283-384 to p-aminobenzoic acid The amino acid supplement (0.02 ml. per ml.), purine supplement, and M/30 sodium citrate were present in all series. Aliquots of the medium were adjusted to pH 5.0, 5.5, 6.0, and 6.5 before autoclaving. In uninoculated control tubes of the four series the pH was 5.2, 5.7, 6.1, and 6.4, respectively, after autoclaving.

positive support for the idea that the mutant is permeable only to the un-ionized form of PABA.

We have also attempted to obtain a more linear response curve at pH 7.0 by the addition to the medium of a trace element mixture (16), 10 γ of xanthopterin per ml., or 0.1 γ of pteroylglutamic acid per ml., or by doubling the concentration of all of the constituents of the medium. None of these additions altered the shape of the response curve.

Activity of Compounds Related to PABA—The ability of mutant 273-384

to utilize compounds related to or containing PABA is indicated by the data of Table II. Amino acids and purines were added to the medium to increase the sensitivity of the test. Inactive compounds included benzoic

TABLE II

Activity of Compounds Related to p-Aminobenzoic Acid*

Compound†	Concentration required for half maximal growth‡
	γ *per ml.*
p-Aminobenzoic acid (PABA)	0.00011
Benzoic acid§	>1000
Aniline§	>1000
o-Aminobenzoic acid§	>1000
m-Aminobenzoic " §	700
p-Hydroxybenzoic acid§	>1000
p Aminophenylacetic acid§	50
p-Aminohippuric acid§	25
2-Amino-5-carboxypyrimidine§	>1000
2-Amino-5-carboxythiazole§	>1000
Pimelic acid§	>1000
p-Aminobenzoylglutamic acid	0.08
Glutamic acid polypeptide of PABA	10
Pteroic acid	0.0015
Pteroylglutamic acid	2.0
Pteroyltriglutamic acid	1.0
Thymine	40 (*Ca.*)

* The amino acid supplement (0.02 ml. per ml.) and the purine supplement were present in all tubes. The medium was initially adjusted to pH 7.0. When necessary, solutions of the materials tested were neutralized with NaOH before addition to the medium.

† The glutamic acid polypeptide of PABA was furnished by Dr. S. Ratner of New York University and the pteroyl compounds by Dr. B. L. Hutchings and Dr. E. L. R. Stokstad of the Lederle Laboratories Division, American Cyanamid Company. The other materials were in general prepared in the Chemical Laboratories of the Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company. We wish to express our gratitude to these people.

‡ The criterion of growth was turbidity readings at 24 hours.

§ These compounds were tested over a range of 0.001 to 1000 γ per ml. both in the presence and absence of 0.0001 γ of PABA per ml. Only benzoic acid was inhibitory within this range. Growth was delayed for 16 hours in the presence of 1000 γ of benzoic acid per ml. but the final growth obtained (40 hours) was not affected.

acid, aniline, o-aminobenzoic acid, p-hydroxybenzoic acid, and the pyrimidine and thiazole analogues of PABA. Tatum and Beadle (6) observed that an *aminobenzoicless* mutant of *Neurospora crassa* grew slowly in the presence of pimelic acid. Our mutant strain of *E. coli* did not attain visible turbidity with added pimelic acid. The slight activity observed with

m-aminobenzoic acid, *p*-aminophenylacetic acid, and *p*-aminohippuric acid could well be due to contamination with traces of PABA. This probably holds as well for *p*-aminobenzoylglutamic acid, which has only 0.14 per cent of the activity of PABA. The sample of pteroic acid contained, as an impurity, 6 per cent of free arylamine (calculated as PABA) and possessed about 7 per cent of the activity of PABA. Pteroylglutamic acid was less than 0.01 per cent as active as PABA. This sample contained 0.2 per cent of free arylamine (as *p*-aminobenzoylglutamic acid). There

TABLE III

Response of Mutant 273-384 to p-Aminobenzoic Acid and to Thymine

Compound		Additions to basal medium*							
		None		Purine supplement		Amino acid supplement†		Amino acid + purine supplements	
		Turbidity readings							
		16 hrs.	64 hrs.	16 hrs	64 hrs.	16 hrs.	64 hrs.	16 hrs.	64 hrs.
	γ *per ml.*								
p-Amino-	0.0005	15	21	4	25	32	34	50	53
benzoic acid	0.01	56	74	68	76	69	74	81	78
Thymine	0.1	0	0			2	3	1	7
	1.0	0	0	0	0	3	4	1	11
	3.0	0	0	0	0	2	5	1	24
	10	2	1			4	8	22	31
	30	1	5	0	0	6	10	44	45
	100	0	2			11	15	53	56
	300	2	8	0	0	7	11	63	67
	1000	0	1	0	0	2	4	27	37
	3000	0	0	0	0	4	5	8	15

* Initially adjusted to pH 7.0.
† 0.02 ml. per ml.

is, therefore, no proof that pteroic acid or pteroylglutamic acid is utilized by the mutant strain.

The response of the mutant to thymine was investigated in more detail (Table III). Thymine is practically inactive alone or in the presence of purines. Light growth occurred in the presence of the amino acids and thymine. The organism was carried through five transfers on this medium. A combination of the amino acids, purines, and thymine effectively replaced PABA. During ten transfers with this combination (30 γ of thymine per ml.) the turbidity obtained increased about 50 per cent. In the experiment shown in Table III the combination supported almost the same turbidity as did excess PABA; however, in some experiments the turbidity

obtained with these supplements has not exceeded half maximum. Concentrations of thymine above 300 γ per ml. appear to be toxic.

We then determined which purines and amino acids were required for growth in the presence of thymine. When thymine and the amino acid supplement were present, the addition of any one of the four purines permitted growth (Table IV). Uracil, cytosine, and xanthopterin were unable to replace the purines. Methionine was an essential component of the amino acid mixture (Table V); however, the addition of methionine alone produced only delayed, submaximal growth. In the presence of methionine, the addition singly of glycine, serine, threonine, lysine, histidine, or tyrosine was stimulatory. Addition of the other amino acids of the mix-

TABLE IV

Response of Mutant 273-384 to Purines and Related Compounds in Presence of Amino Acids and Thymine

Medium adjusted to pH 7.0.   30 γ of thymine per ml. and amino acid supplement (0.02 ml. per ml.) in all tubes.

Additions		Turbidity readings;[*] 16 hrs.
	γ per ml.	
None	10	7
Adenine (A)	10	43
Guanine (G)	10	48
Xanthine (X)...	10	47
Hypoxanthine..	10	46
Uracil (U).. ..	10	5
Cytosine...	10	8
Xanthopterin	10	8
A + G + X + U	5 (Each)	51

* Readings essentially unchanged at 64 hours.

ture singly did not increase the response to methionine. In subsequent tests the growth with a combination of the seven amino acids listed in Table V has equaled that obtained with the entire mixture.

It was observed that the mutant could utilize homocystine in place of methionine in the presence of the other amino acids, thymine, and the purines; however, the final turbidity reached was less than one-half of that with methionine. The relative activity of homocystine and methionine is similar to that previously observed with certain methionine-requiring mutants of *E. coli* (12, 17).

Sensitivity of Mutant 273-384 to Sulfonamides—The growth of mutant 273-384 with PABA, either in the unsupplemented basal medium or in the presence of amino acids and purines, was inhibited by the addition of sulfonamides (Table VI). This inhibition was antagonized by increasing the

concentration of PABA. Pteroylglutamic acid at a concentration of 100 γ per ml. did not possess any demonstrable antisulfonamide activity on either medium when tested alone or in the presence of sufficient PABA to yield partial antagonism. When the combination of amino acids, purines, and thymine was present, the organism attained one-half to two-thirds maximal

TABLE V

Response of Mutant 273-384 to Amino Acids in Presence of Purines and Thymine

Medium adjusted to pH 7.0. 30 γ of thymine per ml. and purine supplement in all tubes.

Experiment No.	Additions per ml.		Turbidity readings	
			16 hrs.	64 hrs.
I	None		0	0
	Amino acid supplement	0.02 ml.	50	49
	" " " *	0.02 "	2	6
	DL-Methionine	10 γ	5	25
		40 "	3	16
		100 "	5	19
	DL-Methionine	10 "		
	+ glycine	20 "	2	39
	+ DL-serine	40 "	23	32
	+ DL-threonine	40 "	25	39
	+ DL-lysine	40 "	25	28
	+ L-histidine	20 "	18	25
	+ L-tyrosine	20 "	23	27
II	Amino acid supplement*	0.02 ml.	2	4
	" " " + 10 γ DL-methionine per ml.		20	42
	Amino acid supplement* + 50 γ DL-methionine per ml.		21	41
	Amino acid supplement* + 50 γ DL-homocystine† per ml.		8	18
	Amino acid supplement* + 100 γ DL-homocystine per ml.		8	17

* Methionine omitted.

† General Biochemicals, Inc.; recrystallized once from water.

turbidity within 24 hours even in the presence of 1280 γ of sulfanilamide or sulfadiazine per ml. Growth was delayed, however, and the final turbidity in the presence of the higher sulfonamide concentrations was suboptimal.

Nutritional Variation of Mutant 273-384—This mutant occasionally grew on the 2nd or 3rd day of incubation in media lacking one or more of the essential supplements (thymine, purines, and amino acids). Certain of

these cultures were purified by plating on A. C. agar (Difco); the single colony cultures thus obtained were transferred ten times under the original conditions and single colony cultures again isolated. The growth requirements of these variants will be summarized briefly. (The concentrations used were PABA 0.02 γ per ml., purines and pyrimidines 10 γ of each per ml., and the amino acids as in the report of Roepke *et al.* (1).)

TABLE VI

Concentrations of Sulfonamides Necessary to Inhibit Growth of Mutant 273-384 in Presence of Various Supplements

PABA	Additions to basal medium		Others	Sulfonamide	Minimal effective concentration of sulfonamide‡		
	Amino acid supplement†	Purine supplement			16 hrs.	24 hrs.	64 hrs.
γ per ml.			γ per ml.		γ per ml.	γ per ml.	γ per ml.
0.002	−	−		Sulfanil-amide	2.5	10	20
0.0002	+	+		"	5	20	40
	+	+	Thymine, 100	"	1–1280§	1280	1280
0.0002	+	+		Sulfa-diazine	0.04	0.08	0.3
0.02	+	+		"	3	3	10
1.0	+	+		"	100		
0.02	+	+	Pteroylglutamic acid, 100	"	3	3	10
	+	+	Thymine, 100	"	160–1280§	1280	1280

* Medium initially adjusted to pH 7.0.

† 0.02 ml. per ml.

‡ Concentration required for half maximal inhibition; *i.e.*, for reduction of the turbidity at a given time to one-half of that obtained in control tubes.

§ Approximately half maximal inhibition was observed over the indicated sulfonamide range.

Strain T grew only in the presence of either PABA or thymine. Strain LD-3 grew either with PABA or with a combination of amino acids and purines. The purine could be xanthine or, less effectively, adenine. In the presence of xanthine, delayed growth occurred with methionine, good growth with a combination of threonine, methionine, cystine, tryptophan, and arginine. Strain B-3 required either PABA or a combination of lysine, methionine, and adenine or xanthine. Strain C-2 was unable to grow in the presence of PABA but grew well when guanine or xanthine was added.

The exact phenomena involved in these variations and their genetic basis are obscure at present. From x-rayed cell suspensions of *E. coli* we have obtained mutants which require the presence of purines or purines and

amino acids for growth but are unable to grow with PABA.[2] These mu-
tants generally can use any of the four purines, although with varying effi-
ciency, and usually require several amino acids. These mutants resemble
in their growth requirements the variant strains derived from mutant
273-384.

Studies with Mutants 45-462 and 1861-460—The nutritional requirements
of mutant 45-462 were studied in detail and appear to be the same as
those of mutant 273-384. The combination of thymine, a purine, and
amino acids was again able to replace PABA. These mutants are cer-
tainly very similar, if not identical.

Mutant 1861-460 grew rapidly in the presence of PABA but was also
able to grow slowly with methionine as the sole supplement. Turbidity was
observed somewhat earlier when both methionine and the purines or the
complete amino acid supplement was present; however, the growth with
such combinations never equaled that observed with PABA. When the
mutant was plated on A. C. agar and single colonies picked, all of the
resulting cultures showed the same characteristics. Thus the strain does
not appear to consist of a mixture of two types of cells but rather to be
analogous to the variant strains of mutant 273-384 described earlier.

DISCUSSION

The experiments reported here provide additional evidence that PABA
(or some derived form) functions in the synthesis of the purines and methio-
nine and indicate a rôle in the formation of thymine and of amino acids
other than methionine. The fact that all three supplements are required
with the unadapted mutant strain is good evidence that the activity of
these materials is not due to contamination with PABA. It should be
emphasized that the mutant, while able to grow in prolonged subculture
with a combination of the three supplements, still grows at a suboptimal
rate and generally does not attain the same maximal turbidity as in the
presence of PABA. This phenomenon appears analogous to the half max-
imal growth obtained with certain organisms if thymine and a purine are
present in place of folic acid (18).

Previous studies of mutant strains of *E. coli* (17, 19) have indicated that
the conversion of cysteine S to the methionine S occurs by the same path-
way as in *Neurospora* (20); *i.e.*,

$$\text{Cysteine} \rightarrow \text{cystathionine} \rightarrow \text{homocysteine} \rightarrow \text{methionine}$$
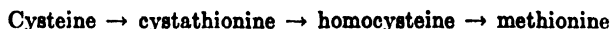
Mutant 273-384 can utilize homocystine, although poorly, for growth in
the absence of PABA but cannot utilize cystine (in the amino acid supple-
ment). This would suggest that PABA, in some form, is involved in the

[2] Roepke, R. R., and Mercer, F. E., unpublished data.

cysteine → cystathionine → homocysteine series of reactions. This question has been studied with mutants of *E. coli* which require the addition of either homocystine or methionine for growth (12). While growth with added homocystine is slower than with added methionine, the concentration of a sulfonamide required to produce half maximal inhibition of growth is approximately the same.[3] Thus the main action of PABA does not appear to be in the methylation of homocysteine but in some prior reaction.[4]

The inactivity of pteroylglutamic acid is surprising, since this compound appears to function in the synthesis of purines and thymine by other organisms. Our parent strain of *E. coli* and mutant 273-384 (growing in the presence of 0.01 γ of PABA per ml.) both synthesize some material which is able to support maximal growth of *Streptococcus faecalis* R on a medium free of pteroylglutamic acid. Miller (22) has demonstrated that sulfanilamide inhibits the synthesis of folic acid by *E. coli;* also Winkler and de Haan (21) have observed that pteroylglutamic acid can act as a sulfonamide antagonist for their strain of *E. coli.* Thus a compound similar to pteroylglutamic acid, but not necessarily identical with it, occurs in *E. coli* and may still be concerned in the syntheses discussed here.

SUMMARY

By irradiation of *Escherichia coli* with x-rays three mutant strains were obtained which grew when *p*-aminobenzoic acid (PABA) was added to the medium. The ability of a series of compounds related to PABA to support growth of mutant 273-384 is reported. The addition of a mixture of purines and amino acids reduces the concentration of PABA required for half maximal growth. PABA can be replaced by a combination of amino acids, a purine, and thymine, although growth is not optimal. The purine requirement can be supplied as adenine, guanine, xanthine, or hypoxanthine. Methionine is an essential component of the amino acid supplement but cannot replace the entire mixture. The data provide additional evidence that PABA functions in *E. coli* in the synthesis of purines and methionine

[3] Lampen, J. O., and Jones, M. J., unpublished data.
[4] Winkler and de Haan (21) have recently studied the ability of many of the compounds used here to antagonize the inhibition of *E. coli* by high concentrations of sulfonamides. They report that homocysteine with or without choline did not replace methionine as an antagonist. One explanation of this difference from our results might be that PABA functions in methionine synthesis at two points; *i.e.*, somewhere in the cysteine → homocysteine series of reactions and in the methylation of homocysteine, the first reaction being inhibited by much lower concentrations of sulfonamide than is the second. We have also observed that the growth with homocysteine is slow and thus may have been missed with the short incubation period used by Winkler and de Haan.

and indicate a rôle in the formation of thymine and of amino acids other than methionine. The point of action of PABA in the synthesis of methionine is discussed.

Both mutant 273-384 and the parent strain of *E. coli* are highly resistant to sulfonamides when growing in the presence of the purines, thymine, and a mixture of amino acids.

Pteroylglutamic acid does not replace PABA for the growth of mutant 273-384.

Mutant 273-384 can give rise to variants which can grow with only two (or even one) of the three supplements it originally requires in the absence of PABA. Certain of these cultures eventually lose their ability to grow in the presence of PABA.

Mutant 45-462 appears to be very similar to strain 273-384. Mutant 1861-460 grows slowly in the presence of methionine alone and appears similar to the variant strains obtained from mutant 273-384.

BIBLIOGRAPHY

1. Roepke, R. R., Libby, R. L., and Small, M. H., *J. Bact.*, **48**, 401 (1944).
2. Roepke, R. R., and Mercer, F. E., *J. Bact.*, **54**, 731 (1947).
3. Woods, D. D., *Brit. J. Exp. Path.*, **21**, 74 (1940).
4. Rubbo, S. D., Maxwell, M., Fairbridge, R. A., and Gillespie, J. M., *Australian J. Exp. Biol. and Med. Sc.*, **19**, 185 (1941).
5. Peterson, W. H., and Peterson, M. S., *Bact. Rev.*, **9**, 49 (1945).
6. Tatum, E. L., and Beadle, G. W., *Proc. Nat. Acad. Sc.*, **28**, 234 (1942).
7. Fries, N., *Nature*, **155**, 757 (1945).
8. Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., SubbaRow, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, **103**, 667 (1946).
9. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, **166**, 435 (1946).
10. Lampen, J. O., and Jones, M. J., *J. Biol. Chem.*, **170**, 133 (1947).
11. Lampen, J. O., Roepke, R. R., and Jones, M. J., *J. Biol. Chem.*, **164**, 789 (1946).
12. Lampen, J. O., Jones, M. J., and Perkins, A. B., *Arch. Biochem.*, **13**, 33 (1947).
13. MacLeod, C. W., *J. Exp. Med.*, **72**, 217 (1940).
14. Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **159**, 273 (1945).
15. Wyss, O., Lilly, V. G., and Leonian, L. H., *Science*, **99**, 18 (1944).
16. Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.*, **150**, 325 (1943).
17. Lampen, J. O., Roepke, R. R., and Jones, M. J., *Arch. Biochem.*, **13**, 55 (1947).
18. Stokes, J. L., *J. Bact.*, **48**, 201 (1944).
19. Simmonds, S., *J. Biol. Chem.*, **174**, 717 (1948).
20. Horowitz, N. H., *J. Biol. Chem.*, **171**, 255 (1947).
21. Winkler, K. C., and de Haan, P. G., *Arch. Biochem.*, **18**, 97 (1948).
22. Miller, A. K., *Proc. Soc. Exp. Biol. and Med.*, **57**, 151 (1944).

THE EFFECT OF THE IONIC ENVIRONMENT ON THE SYNTHESIS OF GLYCOGEN FROM GLUCOSE IN RAT LIVER SLICES

By JOHN M. BUCHANAN,* A. BAIRD HASTINGS, and FRANCES B. NESBETT

(From the Department of Biological Chemistry, Harvard Medical School, Boston)

The effect of the ionic environment of the incubating medium upon glycogen formation by liver slices was first reported by Ostern, Herbert, and Holmes (1). Using rabbit liver slices, they found that, aerobically, calcium stimulated glycogen formation in a modified Ringer's medium containing 1 per cent glucose. Addition of phosphate to this medium resulted in a decrease in glycogen synthesis.

The effect of calcium on glycogenesis by rabbit liver slices has been confirmed in this laboratory. In agreement with other investigators, however, little glycogen synthesis from glucose could be obtained in the presence of rat liver slices incubated in a calcium-containing Ringer's solution. In experiments previously reported, a medium simulating the cationic composition of the intracellular fluid, *i.e.* high in potassium and magnesium, was found to promote glycogenesis by rat liver slices (2).

Since the publication of these preliminary experiments, the ionic composition of the incubating medium has been systematically varied in order to determine the rôle which different inorganic ions play in glycogen synthesis from glucose by rat liver slices. It is the purpose of this communication to report these experiments.

EXPERIMENTAL

Rats were fasted for a period of 18 hours prior to the experiment. Animals were killed by a blow on the head and decapitated to exsanguinate their tissues. The liver was removed and the blood quickly washed away. Slices were cut about 300 to 500 μ thick and washed in their appropriate salt solution (pH 7.5) which was equilibrated with 5 per cent CO_2-95 per cent O_2 at room temperature. The slices were collected on filter paper and about 300 to 500 mg. of tissue were placed in each of several 25 ml. Erlenmeyer flasks containing 2 ml. of experimental medium. These experimental media were in all cases approximately osmolar in respect to ionic composition. When added, glucose was present in concentrations

---

* Present address, Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.

of 1 per cent. The pH values of the media were determined by the method of Hastings and Sendroy (3).

The vessels containing the tissue were placed in a bath at 38° and equilibrated with 5 per cent CO_2-95 per cent O_2 for the first 5 to 10 minutes of shaking. The vessels were then closed and incubation of the tissues continued for 1 hour. At the end of this time, 1 ml. of 80 per cent KOH was added and the vessels heated on a steam bath until their contents were homogeneous. The solution was then transferred to a 15 ml. graduated centrifuge tube and made up to a 5 ml. volume. 1 ml. was taken for total phosphorus determination according to the method of Fiske and Subbarow (4). The remaining 4 ml. were used for glycogen determination, according to the method of Good, Kramer, and Somogyi (5), as modified by Sjögren et al. (6). Standard glucose solutions were made up in 1 M sodium sulfate in order to contain the same amount of salt as the unknown sugar samples from the hydrolyzed glycogen. Glycogen values are expressed in terms of their glucose equivalents, glucose being determined by the Folin method (7). The glycogen content of liver slices is reported in mg. of glycogen glucose per gm. of liver wet weight.

It was found convenient to estimate the wet weight of the liver slices from total phosphorus determinations. The value, 1 mg. of total phosphorus equals 250 mg. of wet weight of rat liver, has been used in the calculations. This figure was obtained by determining the total phosphorus content of unsliced pieces of liver. The initial glycogen and phosphorus contents of the liver were determined before incubation in order to provide a basis for comparison.

Since the magnesium and calcium concentrations in the presence of bicarbonate (40 mM per liter, pH 7.5) in these solutions exceeded the solubility product of calcium and magnesium carbonate, addition of the reagents in the preparation of the media was made in a definite order. Magnesium or calcium was added first as the chloride or carbonate, followed by hydrochloric acid, which was added next to make the solutions definitely acid. These solutions were neutralized by the addition of the proper amount of potassium or sodium bicarbonate. All media were equilibrated with 5 per cent CO_2-95 per cent O_2. No significant precipitation of $CaCO_3$ occurred in supersaturated solutions containing in addition to glucose 10 mM per liter of calcium ions and 40 mM per liter of bicarbonate at pH 7.5 for a period of 4 hours after preparation of the media.

Results

Factors Involved in Glycogen Synthesis by Rat Liver Slices

Prior to a detailed investigation of the effect of individual ions on gly-

cogenesis, the effects of several other factors in glycogen synthesis were determined.

Concentration of Substrate—In confirmation of the work of others (1) it was found that glycogen was not formed from glucose in optimal amounts until a concentration of 1 per cent glucose was reached. Preliminary experiments with concentrations higher than 1 per cent did not yield conclusive evidence that there was any advantage in raising the concentration of glucose above this value.

Duration of Experiment—It was found that glycogen formation from glucose takes place most rapidly during the 1st hour of incubation. In view of this fact, the following experiments in this paper were limited to an incubation period of 1 hour.

TABLE I

Effect of pH on Glycogen Synthesis by Rat Liver Slices

All the solutions contained 1 per cent glucose, 20 mM per liter of Mg^{++}, 130 mM per liter of K^+, 170 mM per liter of total anions (Cl^- and HCO_3^-), and were equilibrated with 95 per cent O_2-5 per cent CO_2. Temperature, 38°, time of experiment, 60 minutes.

HCO_3^- concentration	Initial pH	Final pH	Glycogen	
			Present	Formed
mM per l.			*mg. per gm.*	*mg. per gm.*
10	6.88	6.90	1.7	0.6
20	7.25	7.15	3.3	2.2
40	7.50	7.45	3.8	2.7
80	7.75	7.75	1.4	0.3
Initial glycogen			1.1	

Effect of pH—Six experiments on the effect of pH on glycogen formation from glucose were made. Solutions were prepared having a pH of 6.88, 7.25, 7.50, and 7.75. After incubation of these solutions with 300 to 400 mg. of liver slices for a period of 1 hour, the pH values of the media were respectively 6.90, 7.15, 7.45, and 7.75. In all cases except one, an optimal synthesis of glycogen was obtained by using a medium whose initial pH was 7.50. The data of a representative experiment are recorded in Table I. Since the range of pH for optimal glycogen formation from glucose is limited, the final pH of solutions was determined to insure that the pH of the medium had stayed within the optimal range during the course of the experiment.

Influence of Individual Ions on Glycogen Synthesis from Glucose in Rat Liver Slices—It is to be noted that the net accumulation of glycogen is the result of a balance between two opposing reactions, glycogenesis and

glycogenolysis. In the presence of 1 per cent glucose and suitable salt solution, this balance is in favor of the glycogen-forming reactions, provided the initial glycogen content of the liver is not too high. In the absence of glucose in the incubating medium, however, there may result an over-all breakdown of the preformed glycogen stores of the liver slices. Glycogenesis is then best studied in the presence of glucose, and glycogenolysis in its absence.

"Intracellular" versus "Ringer's" Solution—Before a detailed investigation of the effects of individual ions on the glycogenic and glycogenolytic processes of the rat liver slice was carried out, a comparison was made of the ability of liver slices to synthesize glycogen in media, one of which was composed of "extracellular" ions (*i.e.*, Ringer's solution (Ca^{++} = 1, Mg^{++} = 1, Na^+ = 152, K^+ = 5, HCO_3^- = 40, Cl^- = 121 mM per liter)) and the other a medium which simulated the "intracellular" cationic composition of the tissue fluids (Mg^{++} = 20, K^+ = 130, Cl^- = 130, HCO_3^- = 40 mM per liter). Initial concentrations of 40 mM per liter of HCO_3^- were reduced to 30 to 35 mM per liter after the addition of liver slices.

A comparison of these two solutions for their ability to promote the formation of liver glycogen *in vitro* has been made with the realization that certain data on tissue composition are not available. In the first place, only a preliminary but not thoroughly adequate description of the ionic composition of the intracellular fluid is available. This is in contrast to our knowledge of the nature of the extracellular fluid. In view of the complexity of the anionic pattern of the intracellular fluid, it was practical at this time to study in detail only the effects of the intracellular cations. The two major intracellular cations are magnesium and potassium, which are present in quantities of 45 m.eq. per liter and 155 m.eq. per liter of cell water, respectively (8). There is no definite information given, however, about the relative amounts of these ions which are present in ionic and non-ionic forms. This factor is undoubtedly important in the preparation of a truly intracellular medium, but, in view of the absence of exact knowledge on this point, no allowance has been made for it in the experiments to be described. The results of experiments with rat liver are reported in Fig. 1. As may be seen, there are several experiments (Nos. 4, 5, 6, 7, and 8) in which glycogen was synthesized from glucose in Ringer's solution, but the increase of glycogen with the K-Mg medium was always greater. The average increase in glycogen when a glucose-Ringer's solution was used was 0.5 mg. per gm. of tissue; with a glucose-intracellular solution, 1.7 mg.

The questions of which cations were responsible for this difference in glycogen synthesis and of how variations in the concentrations of indi-
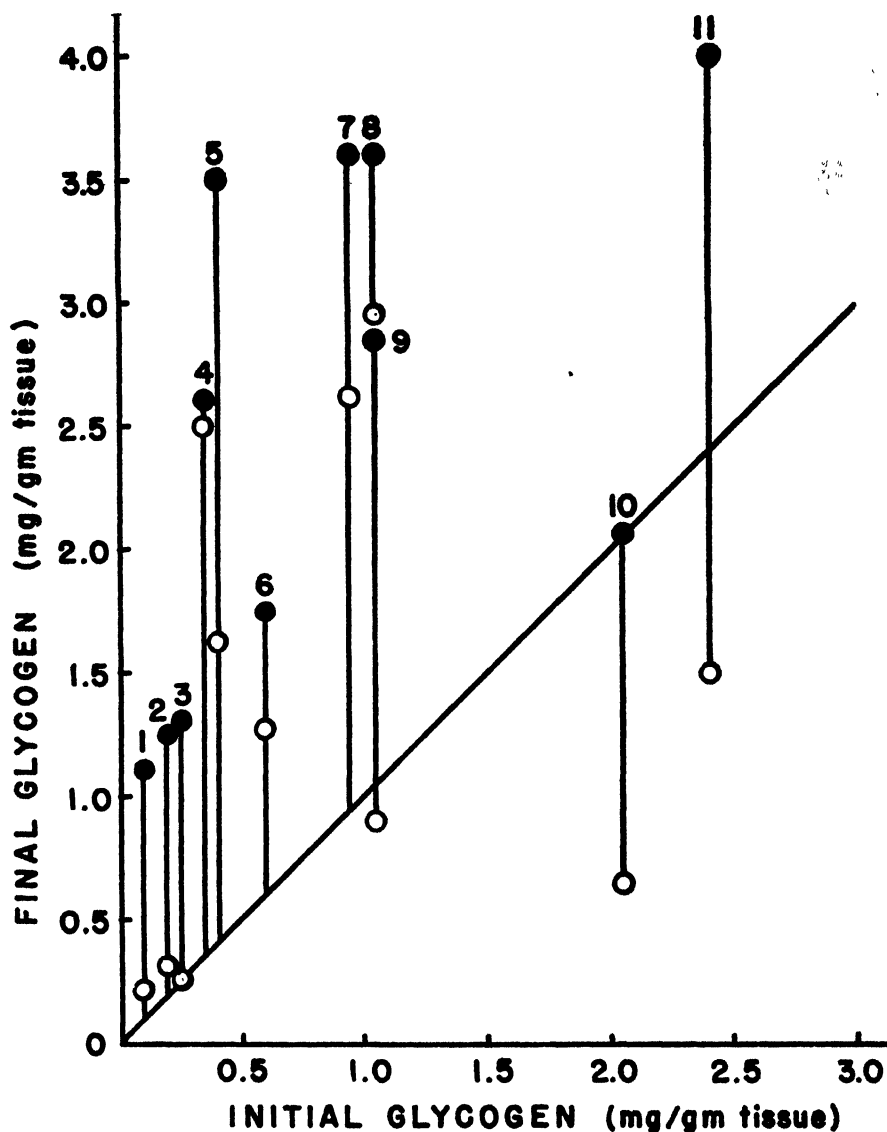
FIG. 1. Comparison of glycogen formation in rat liver slices incubated in Ringer's and "intracellular" media. ●, final glycogen content in the intracellular medium; ○, final glycogen content in Ringer's solution. All the values above the diagonal line represent glycogen synthesis; those below, glycogenolysis. See the text for the ionic composition of the media. All the vessels contained 1 per cent glucose.

vidual cations affected the amount of glycogen synthesized were then explored.

Effect of Sodium and Potassium. (a) *Glycogenesis*—In these experiments media were prepared which contained an unvaried pattern of anions and divalent cation. The variable constituent was the monovalent cation, one medium containing potassium and the other sodium.

As can be seen from the data given in Table II, the nature of the monovalent cation played a large rôle in the glycogenic function of the liver slices. In each experiment, there was a marked increase in the glycogen formed by rat liver slices in the potassium medium over that in the sodium medium.

In order to investigate the possibility that the other cations of the medium could exert an influence on this potassium-sodium effect, these were varied independently. In two experiments not included in Table II

TABLE II

Effect of Sodium and Potassium Ions on Glycogenic and Glycogenolytic Activity of Rat Liver Slices Incubated in Presence and Absence of Glucose

Composition of media, Mg^{++}, 20 mM per liter; monovalent cations, 130 mM per liter; Cl^-, 130 mM per liter; HCO_3^-, 40 mM per liter; pH 7.50; glucose, 1 per cent when present.

cation	Time	Glucose present in medium		Glucose absent in medium	
		Glycogen present	Glycogen formed	Glycogen present	Glycogen formed
	min.	*mg. per gm.*	*mg. per gm.*	*mg. per gm.*	*mg. per gm.*
Na	0	2.0		2.0	
	60	1.7	−0.3	0.6	−1.4
K	0	2.0		2.0	
	60	2.9	+0.9	1.6	−0.4

calcium was substituted for magnesium, and in two other instances calcium and magnesium were used in place of magnesium alone. In none of these cases was the difference between the effects of potassium and sodium altered.

(b) *Glycogenolysis*—There was also a marked difference in the rate of glycogenolysis by liver slices incubated for 1 hour in solutions containing either potassium or sodium, without glucose. When preformed glycogen was present, it was readily broken down in the sodium (glucose-free) solution; but, in the presence of a potassium (glucose-free) medium, liver slices did not appreciably decrease their glycogen stores during the incubation period (Table II).

In an experiment in which half of the monovalent cation present was potassium (72 mM per liter) and the other half sodium (73 mM per liter) and in which calcium was initially present in a concentration of 10 mM

per liter synthesis of glycogen amounting to 1.1 mg. per gm. of tissue was obtained. This suggests that the sodium ion does not interfere with glycogen formation as long as the potassium ion concentration is sufficiently high in the extracellular medium to maintain a high intracellular concentration of potassium.

Effect of Calcium and Magnesium As Source of Divalent Cation—The rôle of divalent ions in glycogen formation from glucose by rat liver is shown in Fig. 2. When magnesium ions were added to the incubating solutions containing potassium ions as the cationic constituent, there was an increase in the amount of glycogen synthesized. It was difficult, however, to establish a magnesium concentration at which this increase in glycogen synthesis was optimal. Since, however, liver slices contain large amounts of intracellular magnesium which are retained by the tissue during preparation of the liver slice for incubation, one might expect that it would be difficult to show marked effects on glycogen synthesis by the addition of magnesium ions to the incubating medium.

The addition of the extracellular cation, calcium, to the medium in optimal concentrations had a more pronounced effect on glycogen synthesis than did the addition of magnesium ions. Seven experiments in which calcium as well as potassium ions were included in the incubating medium are shown in Fig. 2. Initial calcium concentrations of 1, 5, and 10 mM per liter were used. There was a progressive increase in the glycogen formed with increasing calcium ion concentration.

The initial calcium concentrations of 5 and 10 mM per liter in the presence of 40 mM of bicarbonate at pH 7.5 were both greatly in excess of the concentrations which would exist in similar solutions where the calcium ions were in equilibrium with calcium carbonate. At equilibrium, the calcium ion concentration at 38° would be approximately 0.4 mM per liter. Analyses were made, therefore, to determine what the actual calcium concentrations were in the solutions during the experimental period. It was found that, when the initial calcium concentrations were 5 and 10 mM per liter, the concentrations at the end of 75 minutes of incubation with liver tissue were 3.5 and 6.4 mM per liter, at the end of 140 minutes 3.6 and 6.2 mM per liter, respectively. It is seen from these data, therefore, that the solutions in which the liver was incubated were still highly supersaturated with respect to calcium carbonate at the end of the experiments.

It is of interest to note that, when calcium is present in concentrations approximating physiological concentrations, it is not particularly effective in stimulating glycogen formation in rat liver slices. Only when present in considerably higher concentrations does it serve as an effective stimulant to the glycogenic reaction of the liver.

Effect of Inorganic Phosphate—Ostern, Herbert, and Holmes (1) have reported that glycogen synthesis from glucose by rabbit liver slices in
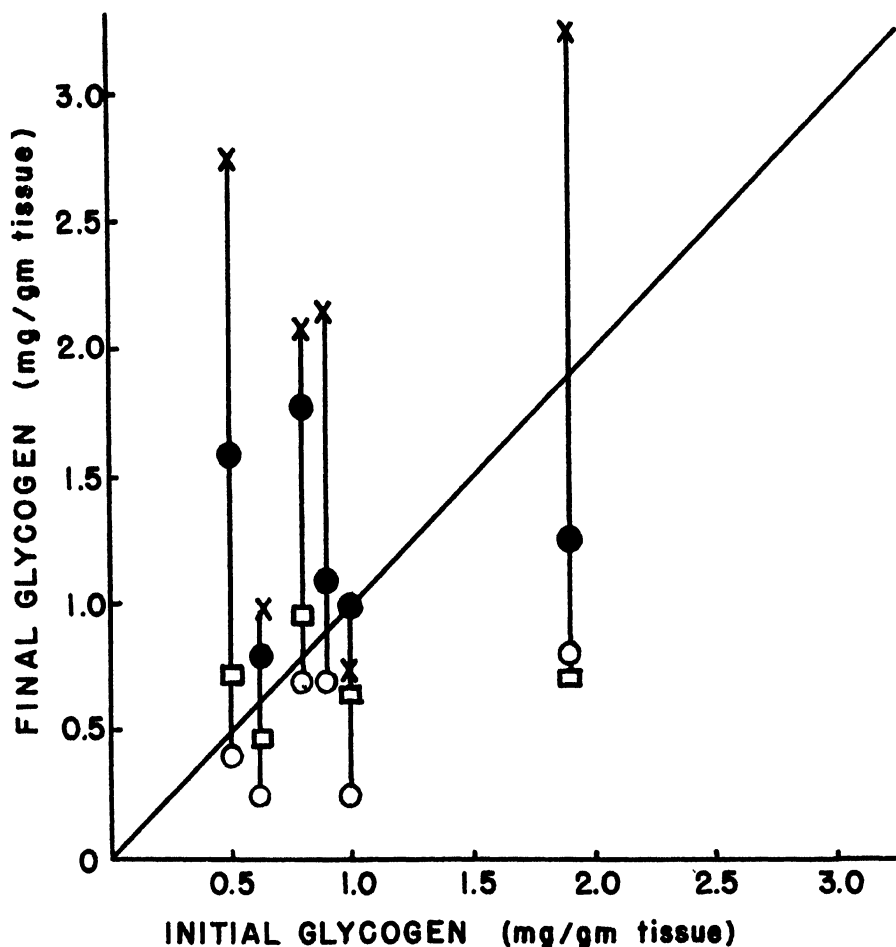
FIG. 2. Effect of calcium concentration on glycogenesis by rat liver slices. Concentration of ions in media expressed as mM per liter. ×, Ca 10, K 150, Cl 130, HCO₃ 40, pH 7.5; ●, Ca 5, K 160, Cl 130, HCO₃ 40, pH 7.5; □, Ca 1.5, K 167, Cl 131, HCO₃ 40, pH 7.5; and ○, Ca 0, K 170, Cl 130, HCO₃ 40, pH 7.5. Time of experiment, 60 minutes; temperature, 38°. All the values above the diagonal line represent glycogen synthesis; those below, glycogenolysis. The solutions contained 1 per cent glucose.

the presence of calcium ions can be reduced by addition of phosphate to the medium. They explained the rôle of calcium by suggesting that it increased the permeability of the cell membrane to glucose. Phosphate

presumably exerted its effect by reducing the calcium ion concentration at the cell membrane.

The results of Colowick and Sutherland (9), using purified enzymes to catalyze the reactions, glucose + ATP → glucose-6-phosphate → glucose-1-phosphate → glycogen + H_3PO_4, suggested that the phosphate effect of Ostern et al. might have an alternative explanation.

In order to achieve glycogen synthesis from glucose-6-phosphate, Colowick and Sutherland (9) added barium ions to reduce the concentra-

TABLE III

Effect of Anions, Phosphate, and Succinate, on Glycogen Formation from Glucose by Rat Liver Slices

K^+ = 130 mM per liter, HCO_3^- = 40 mM per liter; pH 7.5. The remaining anions were Cl^-.

No. of experiments included in average	Concentrations of media components				Time of reaction	Glycogen	
	Succinate	Mg	PO₄	Glucose		Total	Formed
	mM per l.	mM per l.	mM per l.	per cent	min.	mg. per gm.	mg. per gm.
					0	0.23	
5		20	0	0	60	0.20	
		20	0	1	60	0.95	0.72
		20	10	1	60	1.03	0.80
		20	20	1	60	1.06	0.83
3					0	0.17	
		10	0	0	60	0.20	
		10	0	1	60	0.99	0.82
		10	20	1	60	0.94	0.77
		10	40	1	60	0.96	0.79
2					0	0.67	
		20		1	60	2.06	1.39
	87	20		1	60	1.26	0.59
	87	20	10	1	60	0.63	−0.04

tion of inorganic phosphate released by the formation of glycogen from glucose-1-phosphate. In these experiments, the barium ions shift the equilibrium toward glycogen formation by reducing the inorganic phosphate concentration. This observation could well explain the effect of calcium on glycogen synthesis from glucose by liver slices and the effect of inorganic phosphate on this synthesis as reported by Ostern et al. (1).

However, when we studied the effect of inorganic phosphate in experiments in which magnesium alone served as the source of divalent cation, no such reduction of glycogen synthesis with increasing phosphate concentration was found (Table II). By reduction of the millimolar ratio of magnesium to phosphate even to 1:4, such an effect as that reported for calcium by Ostern et al. (1) was not observed.

A striking effect of inorganic phosphate could be observed, however, when glycogen was formed from glucose in the presence of succinate, a member of the tricarboxylic acid cycle. As shown in Table III, the addition of inorganic phosphate in a K-Mg medium containing glucose did not affect the amount of glycogen formed. The addition of succinate, however, did reduce the rate of glycogenesis and, when inorganic phosphate was added, glycogen formation was reduced to zero.

It is improbable that the effect of succinate in reducing glycogen formation from glucose was due to a reduction in the magnesium ion concentration. If this were the case, it would have been expected that inorganic phosphate, when present in concentrations of 40 mM per liter, would have produced a reduction of glycogen synthesis in media containing magnesium ion concentrations of 10 mM per liter. An alternative explanation of the effect of succinate is that it altered the metabolic pathway of glucose metabolism in liver tissue in the direction of glycogenolysis. This might occur in the following manner:

$$\text{glucose}$$
$$\Big\downarrow \text{ATP}$$
$$\text{Glycogen} \longleftarrow \text{glucose-6-phosphate} \xrightarrow{\text{ATP}} \text{fructose-1,6-diphosphate}$$
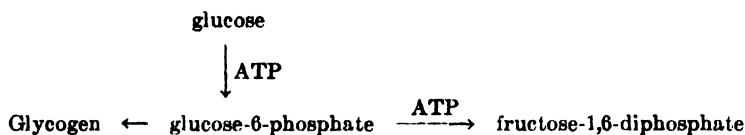
Thus, the oxidation of succinate might result in an "over-phosphorylation" of glucose (10, 11) in rat liver slices, reducing the concentration of glucose-6-phosphate by favoring the production of fructose-1,6-diphosphate and its metabolic products to such an extent that glycogen formation is inhibited.

<center>DISCUSSION</center>

It is interesting to compare the data on the affect of the ionic environment on glycogen formation in liver slices and in diaphragm (12) and heart (13) muscle. In contrast to liver slices, Stadie and Zapp (12) have shown that the presence of potassium ions does not influence glycogen formation when present in amounts less than 75 per cent of the total cations. Above this concentration it is inhibitory. Likewise, the best effects of insulin on the stimulation of glycogen formation in diaphragm muscle were obtained in media lacking in potassium ions. Similar results were obtained by Stadie, Haugaard, and Perlmutter (13) using heart slices. In this tissue the optimal glycogen synthesis from glucose was obtained by using a medium low in ions but high in non-ionic substances such as L-arabinose, D-sorbitol, and D-mannitol. The use of this medium with liver slices caused glycogenolysis rather than glycogenesis. This may have been due to the low pH of the media used by Stadie et al. rather than to the ionic composition.

It is thus apparent that the ionic composition of a medium which causes optimal glycogen formation in one tissue will not necessarily do so in another. Studies on the synthesis of glycogen from both glucose and pyruvate in liver slices have demonstrated, however, the essential rôle of the ions Ca and K in both the glycogenic and glyconeogenic reactions of this tissue.

SUMMARY

Greater glycogen formation by rat liver slices from glucose occurred in a medium rich in potassium than in a medium rich in sodium ions. The presence of the divalent ion, calcium or magnesium, increases glycogen formation, the former being more effective than the latter.

Phosphate had no effect on the rate of glycogen synthesis when magnesium was the divalent cation of the medium. In the presence of succinate, a member of the tricarboxylic acid cycle, the amount of glycogen was reduced, and the simultaneous inclusion of succinate and inorganic phosphate ions lowered the glycogen formation to zero.

For the formation of glycogen from glucose by rat liver slices, the following solution led to the greatest net increase in glycogen deposition: $K^+ = 145$ mM per liter, $Ca^{++} = 10$ mM per liter, $Cl^- = 125$ mM per liter, and $HCO_3^- = 40$ mM per liter, pH 7.5. Glucose was present in concentrations of 1 per cent.

BIBLIOGRAPHY

1. Ostern, P., Herbert, D., and Holmes, E., *Biochem. J.*, **33**, 1858 (1939).
2. Hastings, A. B., and Buchanan, J. M., *Proc. Nat. Acad. Sc.*, **28**, 478 (1942).
3. Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, **61**, 695 (1924).
4. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
5. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol Chem.*, **100**, 485 (1933).
6. Sjögren, B., Nordenskjöld, T., Holmgren, H., and Möllerström, J., *Arch. ges. Physiol.*, **240**, 427 (1938).
7. Folin, O., *J. Biol. Chem.*, **82**, 83 (1929).
8. Hastings, A. B., *Harvey Lectures*, **36**, 91 (1940–41).
9. Colowick, S. P., and Sutherland, E. W., *J. Biol. Chem.*, **144**, 423 (1942).
10. Kalckar, H., *Biochem. J.*, **33**, 631 (1939).
11. Colowick, S. P., Welch, M. S., and Cori, C. F., *J. Biol. Chem.*, **133**, 359 (1940).
12. Stadie, W. C., and Zapp, J. A., Jr., *J. Biol. Chem.*, **170**, 55 (1947).
13. Stadie, W. C., Haugaard, N., and Perlmutter, M., *J. Biol. Chem.*, **171**, 419 (1947).

THE EFFECT OF THE IONIC ENVIRONMENT ON THE SYNTHESIS OF GLYCOGEN AND TOTAL CARBOHYDRATE FROM PYRUVATE IN LIVER SLICES

By JOHN M. BUCHANAN,* A. BAIRD HASTINGS, AND
FRANCES B. NESBETT

(From the Department of Biological Chemistry, Harvard Medical School, Boston)

The synthesis of carbohydrate from pyruvate and lactate has been previously studied *in vitro* in both liver and kidney slices (1–3). Studies of glycogen synthesis from substrates other than hexoses by tissue slices have been fewer and less detailed, however. Cross and Holmes (4) have demonstrated a small glycogen formation by liver slices from lactate and pyruvate with Ringer's solution as the medium in which rabbit liver slices were incubated. These workers found that lactate was converted to glycogen by rabbit liver slices at the rate of 0.3 mg. of glycogen per gm. of tissue per hour, pyruvate at the rate of 0.5 mg. per gm. per hour. In view of the fact that the ionic composition of the nutrient medium plays such an important rôle in glycogen formation from glucose (5), an investigation was made on the rôle of ions on glycogen and total carbohydrate formation from pyruvate in liver slices.

EXPERIMENTAL

Experiments were conducted in a manner identical with those described in the preceding paper (5). Total carbohydrate was determined according to the method of West *et al.* (6), as modified by Benoy and Elliott (7). pH determinations were made colorimetrically according to the method of Hastings and Sendroy (8).

Results

Effect of Ions on Total Carbohydrate Formation in Rat Liver Slices—
Since the effect of ions on glycogen synthesis from glucose had been studied with rat liver slices, rat liver tissue was used in preliminary experiments in which attempts were made to synthesize both carbohydrate and glycogen from pyruvate.

Although the synthesis of carbohydrate from pyruvate by liver slices incubated in the traditional Ringer's solution could be readily confirmed,

---

* Present address, Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia.

it was found that variation of the nature and concentration of the ions of the nutrient medium had a great influence on the amount of carbohydrate formed.

Comparison of Na and K As Monovalent Ion Source—In the first series of experiments two solutions were made up containing Ca + Na and Ca + K, and compared for their ability to promote carbohydrate synthesis in rat liver slices. The data from five experiments were averaged and recorded in Table I. In all the experiments approximately 3 times as much total carbohydrate was synthesized in a medium with sodium as the sole monovalent cation as was synthesized in a corresponding potassium medium.

TABLE I

Comparison of Effect of Na and K Ions on Synthesis of Carbohydrate
from Pyruvate in Rat Liver Slices

All the solutions contained Ca^{++} at 10 mM per liter and either K^+ or Na^+ in concentrations of 120 mM per liter; HCO_3^- = 40 mM per liter; pyruvate 40 mM per liter; 38°; the data are the average of five experiments.

Monovalent cation in medium	Time	Total carbohydrate	
		Present	Formed
	min.	*mg. per gm.*	*mg. per gm.*
Na	0	4.6	
	120	13.2	8.6
K	0	4.7	
	120	7.6	2.9

Comparison of Ca and Mg As Divalent Ion Source—From the data in Table II it is seen that a divalent ion is an essential constituent of the media used in the study of carbohydrate formation. Although magnesium ions in concentrations of 10 mM per liter stimulate total carbohydrate synthesis, calcium, when present in an equal concentration, is much more effective.

Again it must be recalled that, in these experiments, one does not test whether magnesium is essential to the synthesis but whether it is necessary to add it to the environmental medium. A considerable amount of magnesium is present in the tissue itself, and perhaps is there in such amounts that additional magnesium from the medium has a less pronounced effect on carbohydrate synthesis from pyruvate by rat liver slices.

Significant amounts of glycogen were not synthesized from pyruvate in rat liver slices under the conditions of these experiments. In rabbit liver slices, however, both glycogen and carbohydrate were formed from pyruvate, provided the tissue was incubated in a suitable ionic medium.

In order to determine the effect of ions on both these processes, experiments with rabbit liver slices were carried out.

Synthesis of Glycogen and Carbohydrate in Rabbit Liver Slices—Prior to an investigation of the effect of inorganic ions on glycogen formation

TABLE II

Effect of Divalent Cations Ca and Mg on Carbohydrate Synthesis from Pyruvate in Rat Liver Slices

All the solutions contained Na as the monovalent cation. HCO_3^- = 40 mM per liter; pyruvate 40 mM per liter. The data are the average of three experiments. Divalent cations, when added to medium, are present in concentrations of 10 mM per liter.

Divalent cation	Time	Total carbohydrate	
		Present	Formed
	min.	*mg. per gm.*	*mg. per gm.*
	0	10.8	
None	120	12.7	1.9
Mg	120	14.8	4.0
Ca	120	18.7	7.9

TABLE III

Effect of pH on Glyconeogenesis from Pyruvate by Rabbit Liver Slices

The media contained Ca^{++}, 5 mM per liter; K^+ = 105 mM per liter, Na^+ 60 mM per liter, pyruvate 60 mM per liter, HCO_3^- + Cl^- 115 mM per liter. Equilibrated with 95 per cent oxygen-5 per cent CO_2. Time, 90 minutes.

Experiment No.	Initial HCO₃⁻ concentration	Initial pH	Final pH	Glycogen	
				Present	Formed
	mM per l.			*mg. per gm.*	*mg. per gm.*
1	10	6.88	7.17	4.3	3.7
	40	7.50	7.42	6.6	6.0
	80	7.75	7.65	3.0	2.4
	Control			0.6	
2	40	7.50	7.43	3.8	3.3
	80	7.75	7.76	2.6	2.1
	Control			0.5	

from pyruvate, a study was made of the effect of pH, concentration of substrate, and duration of reaction.

Effect of pH—In Table III are included the data of two experiments designed to test the pH at which the optimal rate of glycogen formation occurred from pyruvate. These experiments are only partially complete in view of the fact that, in several instances, there were significant changes

of pH after 2 hours incubation of the rabbit liver slices. Only those data obtained from experiments in which the pH was fairly well controlled have been included in Table III. The data show that lowering or raising the pH significantly below or above 7.50 resulted in a decrease of glycogen formation from pyruvate. In many of the following experiments, pH determinations were made at the conclusion of the experiment to insure that effects observed were not attributable to pH changes of the media during incubation.

Concentration of Substrate—Benoy and Elliott (7) have reported that the concentration of the substrates is a factor influencing carbohydrate synthesis. They obtained carbohydrate synthesis in rat liver and kidney with pyruvate and lactate concentrations of 20 and 41 mM per liter, respectively. Increasing the respective concentrations of these substrates to 100 and 200 mM per liter considerably reduced the synthesis of carbohydrate from these compounds. In our own experiments, it was demonstrated that greater glycogen formation occurred when the pyruvate concentration was 38 mM per liter than when it was 19 mM per liter. No significant difference was found upon raising the pyruvate concentration from 38 mM per liter to 75 mM per liter.

Duration of Incubation—In one experiment, plotted in Fig. 1, the glycogen synthesis from pyruvate was followed for 90 minutes. Tissues were analyzed at intervals of 30 minutes, and the liver glycogen content plotted against time. Within the limits of error, glycogen formation appeared to be a linear function of the duration of the experiment. Likewise, in Experiment 2, where the reaction was allowed to proceed for 210 minutes, the same relation seemed to hold, indicating that the metabolic activity of the tissue continued unchanged for a substantial period of time.

Effect of Ionic Environment on Glycogen and Total Carbohydrate Synthesis. Effect of Divalent Ions, Ca and Mg, on Glycogen Formation—The results of a typical experiment in which the rôle of the divalent ion calcium was tested are shown in Table IV. The inclusion of calcium in the medium in concentrations of 10 mM per liter greatly stimulated glycogen formation by rabbit liver slices. Calcium was in many experiments equally effective at 5 mM per liter, but, if the concentration was lowered to 1 mM per liter, there was very little more synthesis of glycogen than in the control vessel without calcium.

In contrast to calcium, magnesium had little stimulatory effect on glycogen synthesis from pyruvate. This is shown in Table IV.

Effect of Sodium and Potassium on Total Carbohydrate and Glycogen Synthesis—In these experiments, a comparison was made of the ability of three media to stimulate the formation of both total carbohydrate and glycogen from pyruvate during a 2 hour experimental period. All three
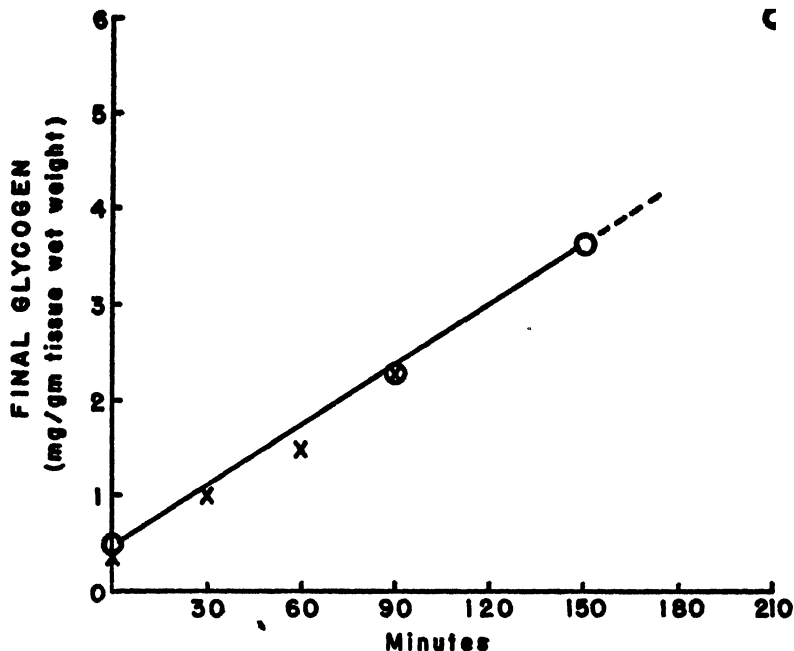
FIG. 1. Performance of the glycogen-forming system with time. $\times$ represents the values of Experiment 1; $\bigcirc$, those of Experiment 2. Composition of media (the values in terms of mM per liter): Experiment 1, $Ca^{++} = 5$, K^+ 105, Na^+ 60, Cl^- 75, HCO_3^- 40, pyruvate 60; Experiment 2, Ca^{++} 10, K^+ 105, Na^+ 60, Cl^- 85, HCO_3^- 40, pyruvate 60.

TABLE IV

Effect of Divalent Ions Ca and Mg on Glycogen Synthesis from Pyruvate in Rabbit Liver Slices

When added, divalent ion is present in concentrations of 10 mM per liter. K^+ = 100 mM per liter, Na^+ 58 mM per liter, HCO_3^- 40 mM per liter, Cl^- 80 mM per liter, pyruvate 58 mM per liter. Time, 120 minutes.

Divalent ion	Glycogen	
	Present	Formed
	mg. per gm.	*mg. per gm.*
Control, zero time...	0.5	
None...	1.8	1.3
Mg...	2.2	1.7
Ca...	5.6	5.1

media contained calcium in concentrations of 10 mM per liter, but varying amounts of sodium and potassium. The monovalent cation composition

of the three media was as follows: Solution 1, K = 145 mM per liter; Solution 2, Na = 145 mM per liter; and Solution 3, K = 73, Na = 72 mM per liter.

The values obtained in three different experiments have been averaged and recorded in Table V. As can be seen, Solution 3 containing both potassium and sodium and Solution 2 containing only sodium were equally effective in stimulating total carbohydrate synthesis. In contrast, in Solution 1, containing only potassium, considerably smaller amounts of total carbohydrate were formed. It is thus apparent that the sodium ion promotes total carbohydrate synthesis in rabbit as well as rat liver slices.

TABLE V

Effect of Sodium and Potassium on Synthesis of Glycogen and Total Carbohydrate from Pyruvate in Rabbit Liver Slices

Composition of media: Ca^{++}, 10 mM per liter, total monovalent cations, 145 mM per liter; HCO_3^- = 40 mM per liter, Cl^- 85 mM per liter, pyruvate 40 mM per liter; pH 7.5. Time, 120 minutes; 38°.

	Glycogen	Total carbohydrate	Glycogen
	mg. per gm.	*mg. per gm.*	*per cent total carbohydrate*
Initial..........................	1.1	5.8	19
Final			
Solution 1, K....................	2.6	7.5	35
" 2, Na..................	2.0	10.3	19
" 3, K, Na............. ..	4.4	9.9	44
Increment			
Solution 1, K...	1.5	1.7	
" 2, Na..................	0.9	4.5	
" 3, K, Na...............	3.3	4.1	

The potassium ions, however, have considerable influence on the type of total carbohydrate formed. In the rabbit tissue slices at zero time, glycogen is 19 per cent of the total carbohydrate present. After incubation with pyruvate in the above three media containing K, Na, and K + Na ions, the glycogen is respectively 35, 19, and 44 per cent of the total carbohydrate present in the tissue. Thus, although considerable total carbohydrate was formed in Solution 2, the ratio of glycogen to total carbohydrate remained the same as in the tissues before incubation. However, in solutions containing potassium ions, the proportion of glycogen to total carbohydrate of the liver slices was considerably increased.

From the data herein presented, it may be concluded that the medium best suited for glycogen and carbohydrate synthesis in rabbit liver slices has the following ionic composition: Ca, 10 mM per liter; K, 73 mM per

liter; Na, 72 mM per liter; HCO_3, 40 mM per liter; pyruvate 40, Cl, 85 mM per liter, equilibrated with 95 per cent O_2-5 per cent CO_2, pH 7.5. In thirty-two experiments performed with this medium, in which the initial glycogen was 1.5 mg. per gm. of tissue or less, glycogen was synthesized from pyruvate at an average rate of 1.5 mg. per gm. of tissue wet weight per hour. This rate is 3 times greater than that reported by Cross and Holmes (4), the incubating medium being the usual Ringer's solution.

DISCUSSION

Deane et al. (9) have reported a cytochemical study of the influence of sodium and potassium ions on glycogen formation in liver slices. They stained transverse sections of rabbit liver slices for glycogen before and after incubation in media of varying ionic composition and containing pyruvate as substrate. Before incubation, glycogen was distributed in a general manner over the transverse section. Regardless of the medium used, however, glycogen disappeared from the interior of the transverse section during incubation to an extent which depended on the initial glycogen content of the liver. When the initial content of glycogen was relatively low, glycogen disappeared entirely from the interior of the section, but there remained a rim of cells at the periphery in which glycogen was present. When liver slices were incubated in a medium containing pyruvate and sodium ions, the glycogen was confined to a rim about eight cells in depth in which the distribution of glycogen was irregular. When potassium was included in the medium as well as pyruvate and sodium, the rim of glycogen-containing cells was of regular distribution and extended toward the interior of the cell for a depth of about twelve cell layers.

Further observations by Deane et al. showed that "at the center of the slice, where no glycogen was present, virtually all mitochondria had disappeared. These alterations in the mitochondria observed after incubation, especially their vesiculation, loss of chromophilia and ultimate disappearance, have been observed repeatedly in dying cells." The degenerative processes of the interior cells were attributed to lack of oxygen and diffusible nutrients, both conditions which would lead to glycogenolysis. It thus seems from these experiments that the anabolic reactions of the liver slice are confined to a layer of cells in the periphery of the liver slice.

Recent experiments by Hastings and coworkers in which rabbit liver slices were incubated with pyruvate and $C^{14}O_2$ have shown that, when the initial glycogen level is relatively low, the specific activity of the glycogen isolated was more or less the same regardless of the ionic composition of

the incubating medium (10). This is probably due to the fact that there is a disappearance of preformed non-isotopic glycogen at the interior of the slice and a synthesis of isotopic glycogen from $C^{14}O_2$ and pyruvate in the peripheral layers of cells. Thus, although the ionic medium does not appreciably influence the specific activity of the glycogen of a liver slice, it does affect the rate of synthesis of total carbohydrate and glycogen in the peripheral layers of cells. It is unknown whether the effects of ions are due to their preservation of normal intracellular enzymatic activity or to their action on specific enzyme systems of the glycogenic process. Regardless of the method of action, calcium probably stimulates both carbohydrate synthesis from pyruvate and glycogen formation from carbohydrate, since (1) the formation of non-glycogen carbohydrate in rat liver slices requires addition of calcium ions and (2) the synthesis of glycogen from glucose is dependent on the calcium concentration.

Sodium and potassium seem to have more specific effects, however. Since addition of the sodium ions to the incubating media stimulates total carbohydrate formation 2- to 3-fold in both rat and rabbit liver slices, and since the addition of potassium ions does not particularly affect the rate of total carbohydrate formation, it may be concluded that sodium ions have a specific effect on the formation of non-glycogen carbohydrate from pyruvate in the peripheral layers of liver cells. However, the presence of potassium ions greatly affects the composition of total carbohydrate formed in the peripheral cells. In the absence of potassium ions, non-glycogen carbohydrate is the principal product formed (81 per cent of the total carbohydrate); in the presence of potassium ions, glycogen is a far larger proportion of the total carbohydrate content of the liver slice. Glycogen formed from pyruvate in the presence of potassium ions is 35 to 44 per cent of the total carbohydrate of the entire liver slice, and probably a much greater percentage of the total carbohydrate of the metabolically active peripheral layer of cells where the newly formed glycogen is localized.

These results concerning glycogen synthesis from pyruvate in rabbit liver slices are in agreement with the studies on glycogen synthesis from glucose in rat liver. In this latter case, as in the present communication, the glycogenic and glycogenolytic processes of the liver slice were dependent on the presence or absence of potassium ions.

SUMMARY

It has been shown that the composition of the ionic environment of the nutrient medium of liver slices plays an important rôle in the rate of synthesis of carbohydrate and glycogen from pyruvate.

In rat liver slices carbohydrate, but not significant amounts of glycogen,

may be formed from pyruvate, provided that calcium and sodium ions are present in the incubating medium in relatively high concentrations.

Rabbit liver slices, however, form both glycogen and non-glycogen carbohydrate from pyruvate. Calcium ions are apparently required in both reactions. Sodium ions again stimulate carbohydrate formation from pyruvate. Although potassium ions do not affect the amount of total carbohydrate formed, they do, however, affect the nature of carbohydrate synthesized. When potassium ions are absent, the main product is non-glycogen carbohydrate but in their presence a considerably greater proportion of glycogen is formed.

Previous experiments are cited which indicate that the glycogenic reactions of the liver slice are confined to a layer of cells in the periphery of the liver slice.

BIBLIOGRAPHY

1. Meyerhof, O., Lohmann, K., and Meier, R., *Biochem. Z.*, **157**, 459 (1925).
2. Takane, R., *Biochem. Z.*, **171**, 403 (1926).
3. Barron, E. S. G., Lyman, C. M., Lipton, M. A., and Goldinger, J. M., *J. Biol. Chem.*, **141**, 957 (1941).
4. Cross, M. C. A., and Holmes, E., *Brit. J. Exp. Path.*, **18**, 370 (1937).
5. Buchanan, J. M., Hastings, A. B., and Nesbett, F. B., *J. Biol. Chem.*, **180**, 435 (1949).
6. West, E. S., Scharles, F. H., and Peterson, V. L., *J. Biol. Chem.*, **82**, 137 (1929).
7. Benoy, M. P., and Elliott, K. A. C., *Biochem. J.*, **31**, 1268 (1937).
8. Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.*, **61**, 695 (1924).
9. Deane, H. W., Nesbett, F. B., Buchanan, J. M., and Hastings, A. B., *J. Cell. and Comp. Physiol.*, **30**, 255 (1947).
10. Hastings, A. B., Solomon, A. K., Anfinsen, C. B., Gould, R. G., and Rosenberg, I. N., *J. Biol. Chem.*, **177**, 717 (1949).

SPECTROPHOTOMETRIC DETERMINATION OF BLOOD OXYGEN*

BY J. B. HICKAM AND R. FRAYSER

(From the Department of Medicine, Duke University School of Medicine, Durham North Carolina)

Gasometric methods for estimating blood oxygen are time-consuming. This consideration often limits the number of observations which can be made in the course of experiments requiring oxygen determinations. In many cases it is an arteriovenous difference which is particularly desired, as in the measurement of cardiac output by the Fick principle, or the estimation of oxygen consumption by human liver, kidney, or brain. The present paper describes a rapid, simple spectrophotometric method for determination of arteriovenous oxygen differences with an accuracy satisfactory for ordinary needs. The absolute oxygen content can also be estimated, subject to error caused by "inactive" hemoglobin. The method is based upon the principle that two well hemolyzed samples of blood drawn nearly simultaneously from the same individual will have substantially the same total hemoglobin content, "inactive" hemoglobin (1), turbidity, and content of other light-absorbing material; and that, consequently, the difference in optical densities of the two samples at an appropriate wave-length will be proportional to the difference in oxyhemoglobin concentration of the two samples. This hypothesis has been tested on samples of blood by comparison of differences in optical density with differences in oxygen content, determined gasometrically by the method of Van Slyke and Neill (2).

The difference in absorption spectra of reduced and oxygenated hemoglobin has been the basis of several methods for determining per cent saturation of blood samples. Much of the basic work in this field was done by Drabkin and his colleagues, who devised a special cell with a short light path, demonstrated the applicability of the Beer-Lambert law to concentrated solutions of hemoglobin and hemoglobin derivatives (3), and measured the per cent of oxygen saturation of undiluted arterial blood samples in man and of circulating arterial blood in the dog (4). Before Drabkin's work Hall had developed a spectroscopic method for determination of per cent saturation of hemoglobin in dilute solutions (5). This was later extended by the use of an ingenious tonometer to measurements of oxygen saturation in whole blood (6). Early work on

this subject was also done by Kramer (7) and by Matthes (8). The Millikan oximeter is based on the same principle (9). Several methods in which photoelectric colorimeters are employed have been described. Brinkman and Wildschut (10) constructed an instrument having a neon lamp to provide light, mostly in the range of 600 to 700 mμ. Using a special cuvette with a short light path, they measured per cent of oxygen saturation in small samples of blood hemolyzed with saponin. The absorption of the sample was compared with its absorption after reduction by $Na_2S_2O_4$, a procedure used earlier by Drabkin. A similar method was described by Jonxis (11), who used a different instrument and a mercury light source with maximum intensity at 436 mμ. Von Issekutz (12) employed a photoelectric colorimeter with a filter which provided light in the near infra-red range and diluted his samples with a relatively large volume of an oxygen-free aqueous solution of saponin and ammonia. After an initial reading the diluted sample was either oxygenated or was reduced by $Na_2S_2O_4$, and the calculation of oxygen content made from the difference in readings. Lowry *et al.* (13) made use of the Evelyn colorimeter with 490 and 620 mμ filters and a specially constructed cell with a short light path. Recently Groom *et al.* (14) has devised a method with a special photometer to make determinations on whole blood contained in transparent plastic tubing.

In most of the methods previously described more or less specialized apparatus is required, particularly cuvettes with a short light path, and small samples of blood were employed. In the present method commercially available equipment and blood samples of 4 to 5 cc. are used.

Equipment and Reagents

1. A Beckman model DU spectrophotometer. The nominal wavelength of 660 mμ was selected. When the instrument is properly focused and used as described, this requires a slit width of about 0.05 mm., resulting in a spread of 1.5 mμ on either side of the nominal wave-length, according to the data submitted by the manufacturers. At this wave-length the extinction coefficient of reduced hemoglobin is approximately 6 times that of oxyhemoglobin. We find the extinction coefficient of reduced hemoglobin to be approximately 0.83 (concentration = 1 mM per liter, depth = 1 cm.) and that of oxyhemoglobin approximately 0.13. These results are in reasonable agreement with the curves published by Horecker (15). At this wave-length the 0.5 cm. cuvettes supplied by the manufacturer provide a suitable light path for the density differences usually encountered.

2. A 30 per cent aqueous solution of saponin, made up every other day and centrifuged to obtain a clear solution. Of the brands used, saponin

(practical, Eastman Kodak Company) has given the most satisfactory results. The solution is neutralized to indicator paper by potassium hydroxide.

3. Mineral oil.

4. Heparin solution, 10 mg. per cc.

5. A 4 per cent aqueous solution of potassium ferricyanide.

6. A 5 per cent aqueous solution of potassium cyanide.

7. Mercury.

8. Glass syringes, Becton, Dickinson Luer-Lok, 2 cc. and 10 cc., assorted hypodermic needles, and syringe caps (soldered needle hubs).

Procedure

Blood samples of 4 to 5 cc. are drawn into oiled, heparinized,[1] 10 cc. Luer-Lok syringes which are then capped with a soldered needle hub. After the volume is noted, mercury is added to assist mixing of blood and heparin, and the syringe is stored in ice water. At least one sample is taken for saturation in air and for total hemoglobin determination. If it is suspected that the hemoglobin concentration has changed during the procedure, samples for saturation and total hemoglobin are taken at appropriate times.

When all samples have been collected, they are hemolyzed by 30 per cent saponin solution added in a volume equal to 20 per cent of the blood sample volume. This is conveniently done by removing the cap from the sample syringe, closing the nozzle by a tightly applied single thickness of longitudinally split rubber tubing, perforating the rubber with a needle attached to a 2 cc. syringe containing the saponin solution, and injecting the required amount of saponin into the sample syringe. The procedure is facilitated by working on a table top. The syringe is recapped and immediately shaken so that agitation of the contained mercury will thoroughly mix blood and saponin. Samples are transferred to the open 0.5 cm. cuvettes through a No. 19 needle attached to the syringe, bent at right angles to avoid injecting mercury from the bottom of the syringe and long enough to reach the bottom of the cuvette. Mineral oil is immediately layered over the blood. The density of each sample is read at 660 mμ, with use of the saturated hemolyzed sample as a blank.

The difference in oxygen content between the saturated sample and any other sample is obtained from the empirically determined formula

Oxygen difference (vol. %) = 7.76 $\times$ optical density difference + 0.36

Arteriovenous difference may be determined by subtracting saturation-arterial difference from saturationvenous difference, or found directly by

---

[1] Enough heparin solution is used to fill the dead space of the syringe.

measuring the density of the venous sample against that of the arterial sample and multiplying the optical density difference by the factor 7.76. The samples being compared must have the same hemoglobin content, but the arteriovenous difference so determined is otherwise independent of the hemoglobin content.

The absolute oxygen content of each sample, if desired, may be estimated on the basis of total hemoglobin content, together with the difference in oxygen content between the sample and the saturated blood. Total hemoglobin is determined by conversion to cyanmethemoglobin, essentially as described by Drabkin (16). 1 cc. of whole blood is added from an Ostwald-Folin pipette to approximately 150 cc. of distilled water in a 200 cc. volumetric flask. 4 cc. of 4 per cent $K_3Fe(CN)_6$ are added, followed after 20 minutes by 1 cc. of 5 per cent KCN. The solution is diluted to volume, and its density is read at 540 mμ in 1.0 cm. cuvettes against a reagent blank. Employing gasometrically determined oxygen capacity as a measure of hemoglobin concentration, Myers[2] has obtained in twelve subjects the value of 11.75 $\pm$ 0.23 (standard deviation) for the extinction coefficient (concentration = 1 mM per liter, depth = 1.0 cm.) of cyanmethemoglobin at 540 mμ. With this value, oxygen capacity in volume per cent is obtained from the density reading of the diluted cyanmethemoglobin solution. 1 mM per liter of hemoglobin is equivalent to 1.67 gm. per cent or to an oxygen capacity of 2.27 volumes per cent. The dilution of the sample is 1:200. Accordingly the oxygen capacity in volume per cent is obtained from the photometer reading by the expression

$$\text{Oxygen capacity} = \frac{\text{optical density} \times 200 \times 2.27}{11.75} = \text{optical density} \times 38.7$$

The oxygen contents of all the samples may now be estimated by subtracting from the oxygen capacity the oxygen difference between the saturated sample and the individual sample, as calculated from the difference in optical densities,

$$\text{Oxygen content} = \text{capacity (colorimetric)} - (7.76 \times \text{density} + 0.36)$$

This determination of absolute oxygen content is subject to whatever error may have been introduced into the colorimetric determination of oxygen capacity by unusual quantities of inactive hemoglobin. The arteriovenous difference, however, is not so affected. In the determination of this quantity by subtraction the value for capacity disappears.

If the hemoglobin concentration of the subject is being changed during the course of a study, the drawing of each sample or set of samples should be accompanied by a further sample for saturation and colorimetric

oxygen capacity. The above procedure for oxygen content is then followed, with use of the appropriate saturation blank and oxygen capacity for each set.

Aside from obvious sources of error, such as inaccuracies in mixing blood and saponin solution, the most serious error results from failure to obtain satisfactory hemolysis. Before reading, all samples must be inspected for clarity by transmitted light. The clarity of solutions will vary somewhat from subject to subject, depending upon the plasma, but in general the spiral filament of a pocket flashlight should be clearly visible through the hemolyzed blood in the cuvette. Old solutions of saponin may give irregular hemolysis. No difficulty has been encountered in the use of Eastman Kodak saponin freshly made up every other day.

We have had no experience with highly turbid bloods, such as those from subjects with leucemia or pathological lipemia.

In the process of hemolysis, it is essential to mix blood and saponin solutions thoroughly immediately after they are brought together. If the solutions are allowed to layer and stand, a protein precipate will form in short order at the interface between the blood and the hypertonic saponin solution. We have found no measurable effect for a period of 1 minute, but by 5 minutes a significant cloudiness will develop. All bloods should be hemolyzed together and the densities determined within 30 minutes after hemolysis. Although density differences may be stable for several hours, samples may begin to show significant changes within 40 minutes after hemolysis. With unneutralized saponin, which yields blood saponin mixtures having a pH in the range of 6.2 to 6.5, the color is less stable. Blood samples with a hemoglobin near the normal range will, however, give the same immediate readings with neutralized or unneutralized saponin. For bloods with a hemoglobin content below 8 gm. per cent we have used neutralized 15 per cent saponin solution.

Verification of Method

In order to determine the increment in optical density for a given change in oxygen content of the sample, the following procedure was adopted. 90 to 100 cc. of human blood were drawn from an antecubital vein into an oiled syringe containing enough heparin solution to fill the dead space. Mercury was added to assist in mixing. A portion of the blood was saturated in air. The oxygen contents of the saturated and partially reduced bloods were determined by the method of Van Slyke and Neill (2). Analyses were done in duplicate and were required to check within 0.1 volume per cent. Several mixtures of these bloods containing graded quantities of the oxygenated and partially reduced components were made in oiled 10 cc. syringes of the Luer-Lok type. It was found con-

TABLE I

Determination of Arteriovenous Blood Oxygen Differences

Subject No.	Oxygen capacity	Oxygen difference, (saturated) − (sample)	Optical density* difference at 660 mμ, (sample) − (saturated)	Subject No.	Oxygen capacity	Oxygen difference, (saturated) − (sample)	Optical density* difference at 660 mμ, (sample) − (saturated)
	vol. per cent	vol. per cent			vol. per cent	vol. per cent	
1	20.04	0.82	0.085	6	18.55	1.07	0.080
		1.63	0.194			2.15	0.216
		2.45	0.270			3.22	0.348
		3.27	0.363			4.29	0.499
		4.08	0.495			5.37	0.615
		4.90	0.603			10.72	1.363
		5.71	0.704	7	17.58	1.17	0.138
		8.17	0.982			2.34	0.260
2	18.78	1.52	0.190			3.50	0.417
		4.55	0.560			4.66	0.566
		6.06	0.718			5.83	0.703
		7.58	0.956			11.68	1.419
		9.10	1.135	8	18.40	1.20	0.123
		15.16	2.010			2.39	0.265
3	17.40	1.15	0.130			3.59	0.409
		2.29	0.258			4.79	0.581
		3.44	0.429			5.98	0.737
		4.58	0.555			11.97	1.488
		5.73	0.728	9	16.88	1.00	0.065
		11.46	1.457			1.98	0.208
4	19.78	1.47	0.151			2.97	0.312
		2.94	0.300			3.96	0.428
		4.42	0.520			4.95	0.559
		5.89	0.630			9.91	1.174
		7.36	0.858				
		14.73	1.780				
5	17.12	0.66	0.033				
		1.32	0.114				
		1.98	0.203				
		2.65	0.285				
		3.31	0.368				
		6.62	0.802				

Regression curve, oxygen difference = 7.76 (optical density difference) + 0.36. Standard deviation from regression, 0.23 volume per cent.

* Depth = 0.5 cm.

venient first to place the desired quantity of saturated blood in the mixture syringe, carefully expel air bubbles, and then to add the reduced blood. This was done by injecting through a rubber membrane, as described above. In this way four to six intermediate mixtures were prepared from

the two samples on which gasometric analyses had been done. The oxygen contents of the mixtures were calculated from the proportions of their components. All bloods were then hemolyzed in the manner already described. The saturated sample was used as a blank against which the density of the other samples was read. The data so obtained on 56 original samples and mixtures from nine subjects are presented in Table I. The regression curve calculated from these data together with the individual points appears in Fig. 1. If Y represents difference in oxygen content in volumes per cent between a saturated sample and any other
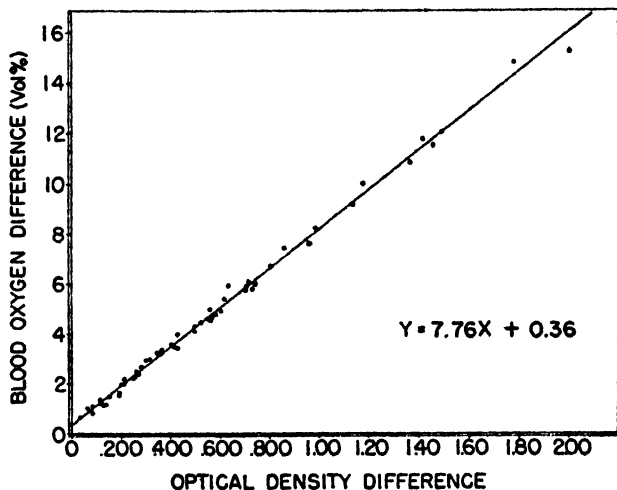
Fig. 1. Each point represents the difference between a partially reduced and a saturated blood sample from a given subject. Oxygen differences are expressed in volumes per cent and were determined on a gasometric basis (see the text). Optical density differences are as read directly from the spectrophotometer at 660 mμ, the saturated sample being used as a blank. There are 56 determinations, representing all data from nine consecutive subjects. The standard deviation from regression is 0.23 volume per cent.

sample from the same subject, and X represents the optical density as read on the machine, the equation for the regression line becomes $Y = 7.76X + 0.36$. The standard deviation from regression is 0.23 volume per cent. Since one is dealing with the difference in oxygen content of two samples, a maximum variation of 0.2 volume per cent would be expected if the individual samples were analyzed gasometrically in duplicate with checks to 0.1 volume per cent.

This agreement with the gasometric method for arteriovenous differences is considered satisfactorily close, particularly in view of the possibilities for error in the verifying procedure described above. These

include inaccuracies in mixing,[3] addition of oxygen dissolved in the saponin solution,[4] and possible changes in total and inactive hemoglobin during the saturation procedure. Prior to hemolysis, blood was stored in ice for periods up to 4 hours. All readings were completed within 30 minutes after hemolysis.

It will be noted that the line in Fig. 1 does not pass through the origin, but intercepts the ordinate at a height of 0.36 volume per cent. This is apparently the effect of physically dissolved oxygen which is present to a greater extent in the saturated sample than in others and which affects the gasometrically determined oxygen differences but not the optical densities. If the saturation values are neglected and oxygen differences and optical density differences are measured with reference to the prepared mixtures having the greatest oxygen contents (0.7 to 1.5 volumes per cent below capacity), the calculated regression line has the formula, $Y = 7.80X + 0.14$. The result agrees with the expectation that dissolved oxygen should exert less effect when the saturated samples are not used.

<center>DISCUSSION</center>

As described above, the method has been in routine use for several months in this laboratory for the determination of blood oxygen contents and arteriovenous differences. This period has covered somewhat more than 300 blood oxygen determinations. In analyzing multiple blood samples the method in our hands has been 5 to 6 times as fast as conventional gasometric procedures.

Of particular interest is the finding that determinations by this method have shown remarkable constancy in per cent oxygen saturation of arterial samples successively drawn from resting normal subjects over a period of 1 to 2 hours. In our experience fluctuations in this measurement in normal subjects are occasionally seen with gasometric techniques and usually indicate technical difficulties.

It should be emphasized that the method depends upon measuring optical density differences between blood samples having the same hemoglobin concentration.

The hemoglobin concentration of normal subjects can undergo very significant changes within a few hours in the course of ordinary daily activities (17). It can be altered markedly in a brief time by changes

---

[3] A few calibrations have indicated that the volume marks on Becton, Dickinson Luer-Lok syringes are sufficiently accurate for the present purpose.

[4] Analysis of the saponin solution has yielded values of approximately 0.4 volume per cent of oxygen. The effect of this quantity, mixed with 5 times its volume of blood and added to all the samples, has been neglected.

in body position and by exercise (18). It may be affected by drugs, such as epinephrine. It varies with local factors, such as venous stasis. In using the method these variations must be kept in mind. When the hemoglobin concentration changes, a fresh sample for saturation must be taken, together with the samples on which oxygen contents are to be determined. Similarly, arteriovenous differences can be determined directly only by comparing samples which have the same hemoglobin concentration.

In adopting such a method, it is apparent that the factors for one's own spectrophotometer as well as the general technique should be checked by gasometric analysis.

SUMMARY

1. A spectrophotometric method is described for the determination of arteriovenous blood oxygen differences and the estimation of blood oxygen contents.

2. The accuracy is satisfactory for most purposes. In determination of arteriovenous differences which have been checked by gasometric analysis, the method has yielded a standard deviation from regression of 0.23 volume per cent.

3. The advantage of the method lies in the speed with which multiple samples can be handled.

BIBLIOGRAPHY

1. Ammundsen, E., and Trier, M., *Acta med. Scand.*, **101**, 451 (1939).
2. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry; Methods, Baltimore, 321 (1932).
3. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112**, 105 (1935–36).
4. Drabkin, D. L., and Schmidt, C. F., *J. Biol. Chem.*, **157**, 69 (1945).
5. Hall, F. G., *J. Physiol.*, **80**, 502 (1934).
6. Hall, F. G , *J. Biol. Chem.*, **130**, 573 (1939).
7. Kramer, K., in Abderhalden, E., Handbuch der biologischen Arbeitsmethoden, Berlin and Vienna, Abt. V, Teil 8, 1083 (1935); quoted by Brinkman and Wil⸍schut (10).
8. Matthes, K., *Arch. exp. Path. u. Pharmakol.*, **176**, 683 (1934).
9. Millikan, G. A., *J. Physiol.*, **79**, 152 (1933).
10. Brinkman, R., and Wildschut, A. J. H., *Acta med. Scand.*, **94**, 459 (1938).
11. Jonxis, J. H. P., *Acta med. Scand.*, **94**, 467 (1938).
12. Von Issekutz, B., Jr., *Arch. exp. Path. u. Pharmakol.*, **197**, 332 (1940–41).
13. Lowry, O. H., Smith, C. A., and Cohen, D. L., *J. Biol. Chem.*, **146**, 519 (1942).
14. Groom, D., Wood, E. H., Burchell, H. B., and Parker, R. L., *Proc. Staff Meetings Mayo Clin.*, **23**, 601 (1948).
15. Horecker, B. L., *J. Biol. Chem.*, **148**, 173 (1943).
16. Drabkin, D. L., and Austin, J. H., *J. Biol. Chem.*, **112**, 51 (1935–36).
17. McCarthy, E. F., and Van Slyke, D. D., *J. Biol. Chem.*, **128**, 567 (1939).
18. Lange, H. F., *Acta med. Scand.*, suppl. 176 (1946).

THE NUCLEIC ACID CONTENT OF FETAL RAT LIVER*

By I. GESCHWIND† AND CHOH HAO LI

(From the Institute of Experimental Biology, University of California, Berkeley)

It has been shown by various investigators that embryonic tissues are richer in nucleic acid than are adult tissues (1). The distribution of the two types of nucleic acids (ribonucleic acid (RNA) and desoxyribonucleic acid (DNA)) is also known to be different in embryonic and adult tissues and in different tissues. For instance, muscle and heart are low in ribonucleic acid concentration, whereas liver and pancreas have a high content of this nucleic acid. The ratio of RNA to DNA is smaller in the liver of sheep embryos than in that of the adult animal. Caspersson and others (2) have suggested that these changes are in some way related to protein synthesis for purposes of growth or of secretion.

Systematic determinations of the changes in nucleic acid concentration in embryonic mammalian liver have not been conducted. Masing (3), working with rabbit liver, found that a decrease in the nucleoprotein phosphorus per unit of nitrogen of the liver occurred from the 4th week of gestation to the adult period; the ages of the animals were, however, not accurately determined. Dumm (4) showed a decrease of "residual phosphorus" in the fetal liver of rats during the later period of gestation; the author did not determine separately the RNA-P and DNA-P. Dumm's results, expressed as residual phosphorus, may be accepted as being due to nucleoprotein phosphorus, because the phosphoprotein phosphorus (the only other phosphorus fraction present in residual phosphorus) is always small in comparison with that for nucleic acids.

In the present experiments fetal rat livers from the 16th to the 21st prenatal day were employed. The nucleic acids in the tissue were fractionated into RNA and DNA. It will be seen that the ratio RNA:DNA is relatively constant from the 16th to the 19th day and that it increases at the 20th day.

EXPERIMENTAL

Pregnant rats of the Long-Evans strain were used; the dates of breeding were accurately determined by the presence of sperm in the vagina.

The animals were anesthetized with nembutal; the fetuses were quickly removed from the uterus and weighed after being dried on filter paper. The livers were extirpated and immediately frozen in a weighing bottle with an acetone-dry ice mixture. Between the time of the death of the animal and the preparation of the livers for nucleic acid determinations, the tissues were covered and stored in the ice chest of a refrigerator. This interval was never greater than 1 hour.

The methods for nucleic acid determinations were modeled after those of Schmidt and Thannhauser (5) and of Schneider (6). The method of Fiske and Subbarow (7) was employed for phosphorus analysis.

TABLE I

Nucleic Acid Content in Fetal Liver of Rat

Day of gestation	No. of pregnant rats	No. of fetuses	Total weight of fetus	Total weight of liver	Ratio, $\frac{\text{Liver}}{\text{Fetus}}$	No. of determinations	P per 100 gm. dry extracted liver		Ratio, $\frac{\text{RNA-P}}{\text{DNA-P}}$
							RNA	DNA	
days			*gm.*	*mg.*			*mg.*	*mg.*	
16th	15	134	0.50	26	0.052	7	1109 ± 59*	1129 ± 38*	0 98
18th	12	105	1.60	95	0.060	5	973 ± 63	1143 ± 67	0.85
19th	7	58	2.10	140	0.067	3	1137 ± 19	1223 ± 9	0.93
20th	9	80	2.90	175	0.060	6	988 ± 20	690 ± 27	1.43
21st	3	25	4.50	245	0.055	1	854	441	1.94

* Mean ± standard error.

Results

As is shown in Table I, the fetal body weight increases steadily throughout the period studied. It is of interest to note that the values are higher than those of Dumm (4), whose data agree with those reported by Donaldson (8). This is probably due to the improved nutritional state of our animals. There is a slight increase in the liver weight, as expressed in percentage of body weight from the 15th to the 19th day of gestation, followed by a slight decrease until about the day of birth, after which a sudden drop occurs.

The last three columns in Table I give the values for RNA-P, DNA-P, and their ratios. Assuming a molecular weight for RNA of 1303 and of 1253 for DNA, the amount of phosphorus is 9.52 and 9.89 per cent respectively; so that if equal moles of RNA and DNA are present, the ratio will be 0.96. It may be seen that both RNA-P and DNA-P are relatively constant from the 16th prenatal day to the 19th, and the ratios suggest that they are present in 1:1 molar relationship. The mean value for RNA-P in this period is 1073 mg. of P per 100 gm. of extracted dry tissue and that for DNA-P 1165 mg. of P.

There is a gradual fall of the RNA content from the 20th prenatal day to 40 days after birth (Table II). The changes in the DNA-P are more striking. Following the 19th prenatal day, a precipitous fall[1] in the level of DNA occurs, changing from 1223 mg. to 441 mg. per cent just before the day of birth. The more gradual decrease from the 1st day of birth to 40 days of age is still more rapid than the rate of decrease of RNA during the same period (Table II).

The marked variation in the ratio of RNA-P to DNA-P observed from the 20th prenatal day to the 5th day after birth is not significant, and the ratio is about 1.45. The value obtained for the liver of 40 day-old male rats is 2.83.

TABLE II

Nucleic Acid Content of Livers of Male Rats

Age	No. of rats	Average body weight	Average liver weight	Ratio, Liver / Body weight	No. of determinations	P per 100 gm. dry extracted liver		Ratio, RNA-P / DNA-P
						RNA	DNA	
days		*gm.*	*gm.*			*mg.*	*mg.*	
1	12	6.1	0.31	0.051	1	672	469	1.43
5	12	12.9	0.45	0.035	2	602	370	1.62
40	4	115.0	4.60	0.040	2	532	188	2.83

DISCUSSION

Davidson (1) has summarized the data obtained by different authors for the nucleic acid content of rat liver and other tissues. The ratio RNA-P:DNA-P for rat liver was found to vary from 1.9 to 4.6, depending upon the method of determination, age, nutritional condition, etc. The same author also obtained a value of 2.2 for fetal rat liver (age?), compared to an adult value of 4.0. In an earlier paper, Davidson and Waymouth (9) reported a ratio of 1.5 for the liver of rat fetuses 1 to 2 days before parturition, with a value of 4.3 for the adult animal. The fact that the livers of rat fetuses have a smaller RNA-P:DNA-P ratio than that for adult tissue is confirmed by the present experiments. Our value for the fetal liver of rats from the 16th to the 19th prenatal day is about 0.92, and it increases to 2.83 for the liver of rats 40 days of age.

Schneider (10) has determined the RNA-P:DNA-P ratio for rat liver tumors and compared it with that found in adult rat liver. It was found that the ratio for tumor tissue is much lower than non-tumor liver, the

[1] This decrease in DNA-P is less striking if the total amount of DNA-P present is considered. The authors are grateful to the referee who pointed out that a large proportion of this drop is undoubtedly due to the increase in the cytoplasm and the proportionate decrease of the nuclear volume as the mitotic rate decreases.

value for the tumor being 0.81, in contrast to the normal ratio of 2.49. It appears that the rapidly growing livers, either of tumor or fetal origin, possess a nearly equal molar quantity of DNA and RNA.

The partition of nucleic acids in the regenerating rat liver has been recently investigated (11, 12). Novikoff and Potter (11) found that there is an apparent correlation between the period of rapid growth and the increase in RNA concentration. No consistent changes in DNA content occurred in the regenerating period. Our results, as shown in Fig. 1, indicate more marked changes in the concentration of liver DNA from the prenatal to the postnatal period than in the values for RNA. It is
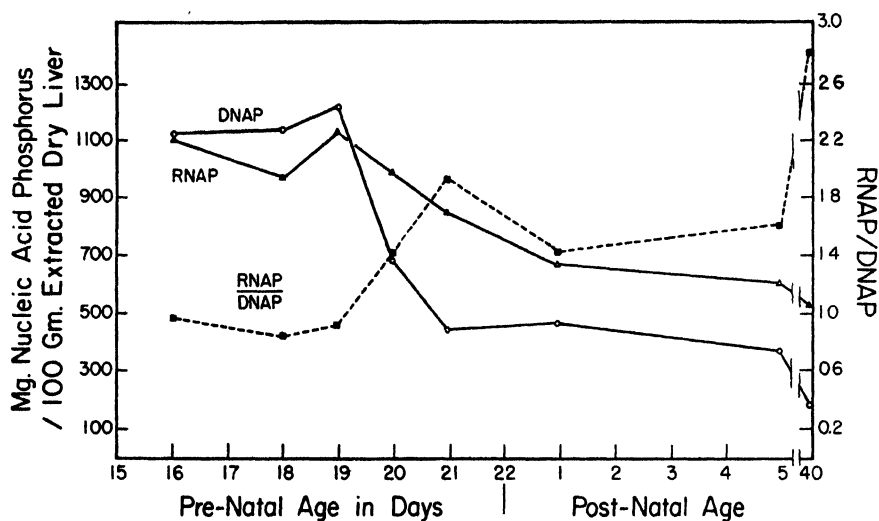
Fig. 1. Changes in the ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) phosphorus of rat liver from the 16th day prenatal to 40 days after birth.

clear, however, that the fetal liver has the highest concentration of total nucleic acids during the most rapid growing period of the tissue from the 16th to the 19th prenatal day.

SUMMARY

1. The concentration of ribonucleic (RNA) and desoxyribonucleic (DNA) acid phosphorus in the liver of rat fetuses has been determined.

2. The results demonstrate a constant RNA content from the 16th to the 20th prenatal day, followed by a gradual decrease to the juvenile level. The concentration of DNA-P is constant from the 16th to the 19th day, followed by a decrease to the juvenile level.

3. The ratio RNA-P:DNA-P has been shown to be relatively constant

at about 0.9 from the 16th to the 19th day; it then increases gradually, a value of 2.9 being found at 40 days of age.

BIBLIOGRAPHY

1. Davidson, J. N., *Cold Spring Harbor Symposia Quant. Biol.*, **12**, 50 (1947).
2. Caspersson, T., *Symposia Soc. Exp. Biol.*, **1**, 127 (1947).
3. Masing, E., *Z. physiol. Chem.*, **75**, 135 (1911).
4. Dumm, M. E., *J. Cell. and Comp. Physiol.*, **21**, 27 (1943).
5. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, **161**, 83 (1945).
6. Schneider, W. C., *J. Biol. Chem.*, **164**, 747 (1946).
7. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
8. Donaldson, H. H., The rat, Memoirs of The Wistar·Institute of Anatomy and Biology, Philadelphia (1945).
9. Davidson, J. N., and Waymouth, C., *Biochem. J.*, **38**, 379 (1944).
10. Schneider, W. C., *Cold Spring Harbor Symposia Quant. Biol.*, **12**, 169 (1947).
11. Novikoff, A. B., and Potter, V. R., *J. Biol. Chem.*, **173**, 223 (1947).
12. Drabkin, D. L., *J. Biol. Chem.*, **171**, 395 (1947).

RESOLUTION OF RACEMIC PHENYLALANINE, TYROSINE, AND TRYPTOPHAN

By JAMES B. GILBERT, VINCENT E. PRICE, AND JESSE P. GREENSTEIN

(From the National Cancer Institute, National Institutes of Health, Bethesda, Maryland)

A simple method of resolution of racemic alanine, methionine, leucine, valine, threonine, serine, isoleucine, and aspartic and glutamic acids has been described (1, 2). This is based upon the asymmetric enzymatic hydrolysis of the N-acylated derivatives of these amino acids by a purified enzyme preparati on from hog kidney. Complete digestion of such derivatives yields a mixture of free L-amino acid and N-acylated D-amino acid which can be r eadily separated by virtue of the alcohol solubility of the latter and the alcohol insolubility of the former. Acid hydrolysis of the N-acylated D-amino acid followed by neutralization yields the free D-amino acid.

The hog kidney preparation acts extremely slowly on N-acylated derivatives of phenylalanine and tryptophan (1). For the resolution of the racemic aromatic substituted amino acids, therefore, it was decided to employ a purified carboxypeptidase preparation from fresh beef pancreas prepared according to Anson (3).; With this preparation, and with chloroacetylated racemic phenylala ne, tyrosine, and tryptophan as substrates, a rapid separation and isolation of the pure optical isomers of these amino acids have been achieved.

EXPERIMENTAL

Carboxypeptidase Preparation—The enzyme used in these studies was prepared from 10 kilos of frozen beef pancreas (3). The ground tissue was extracted with 2 per cent sodium chloride and toluene, and the skimmed extract filtered and treated with acetic acid to pH 4.7. The mixture was filtered and treated with solid ammonium sulfate to about half saturation. The precipitate was filtered and dialyzed against cold water. The dialyzed mixture was centrifuged, the supernatant discarded, and the precipitate suspended in cold distilled water to make a final volume of 330 cc. This enzyme preparation was stored at 5°, and, when employed for the digestions, was brought into solution by adding the minimum amount of solid lithium chloride.

An aqueous extract of the frozen pancreas tissue hydrolyzed chloroacetyl-DL-phenylalanine, chloroacetyl-DL-tyrosine, and chloroacetyl-DL-

tryptophan at rates, respectively, of 117, 81, and 68 μM per hour per mg. of N at 37°. The digests were composed of 1 cc. of aqueous extract equivalent to 330 mg. of tissue, 2 cc. of 0.06 M phosphate buffer at pH 7.5, and 1 cc. of water or 0.5 M neutralized substrate solution. The corresponding rates for the purified carboxypeptidase preparation, with 1 cc. of a 1:30 dilution of the preparation in digests similar to those described for the crude tissue, were 1933, 1240, and 1060. On the basis of total N, therefore, the purified enzyme was 15 to 17 times as active as the original tissue. From these data, the amount of enzyme required to hydrolyze the larger batches of chloroacetylated racemic amino acids in the resolution procedure can readily be calculated.

The rates of hydrolysis by the purified carboxypeptidase preparation of acetyl-DL-phenylalanine and of acetyl-DL-tryptophan are extremely low, being respectively 14 and 6 μM per hour per mg. of N. The considerable difference in susceptibility of the chloroacetyl and acetyl derivatives of these amino acids stands in contrast to the relatively smaller difference in susceptibility of the chloroacetyl and acetyl derivatives of the aliphatic amino acids toward hog kidney enzyme preparations (1, 2). In the latter case, the chloroacetyl derivatives of alanine, leucine, valine, etc., are hydrolyzed at rates about 4-fold those of the corresponding acetyl derivatives.

These data are in accord with the observations of several investigators (4–8), which demonstrated that the rate of carboxypeptidase activity on susceptible substrates varied considerably with the nature of the acyl radical and of the terminal amino acid residues. Of even more decisive concern in the resolution of the racemic amino acids is the stereochemical specificity of pancreatic carboxypeptidase (6–8) whereby only those peptides containing a terminal amino acid of the natural L configuration are hydrolyzed by the enzyme. Thus, carbobenzoxyglycyl-D-phenylalanine, in contrast with the corresponding L isomer, is completely resistant to pancreatic carboxypeptidase (7, 8). Fig. 1 shows that the D isomers of chloroacetyl-DL-phenylalanine and of chloroacetyl-DL-alanine are not attacked even after many hours from the time at which the L isomer is completely hydrolyzed by pancreatic carboxypeptidase and by hog kidney respectively.

Resolution Procedure—The method of resolving the three amino acids follows substantially that outlined in a previous paper (2). Finely divided and pure chloroacetyl-DL-phenylalanine (1), tyrosine (m.p. 158°, N found 5.4, calculated 5.4), and tryptophan (1) are suspended in a little cold water and brought into solution at pH 7.6 by cautious addition of 6 N lithium hydroxide. To this solution is added enough enzyme solution to hydrolyze the L isomer in 2 hours at 37°. The digests generally consisted of 0.25 to 0.5 mole of substrate dissolved in 300 to 700 cc. of solution.

The enzyme solution added varied from 20 to 60 cc. In order to insure complete hydrolysis, the digests are usually set up in the late afternoon and allowed to stand at 37° until early the next morning. The L-amino acid crystallizes in high yield and customarily fills the digestion flask. The mixture is brought to pH 5.0 by addition of glacial acetic acid, the L-amino acid filtered by suction, and washed with cold water.

From 170 gm. of chloroacetyl-DL-phenylalanine, 150 gm. of chloroacetyl-DL-tyrosine, and 82 gm. of chloroacetyl-DL-tryptophan the following amounts of crude products were obtained: 42 gm. of L-phenylalanine, 42 gm. of L-tyrosine, and 22 gm. of L-tryptophan. After recrystallization
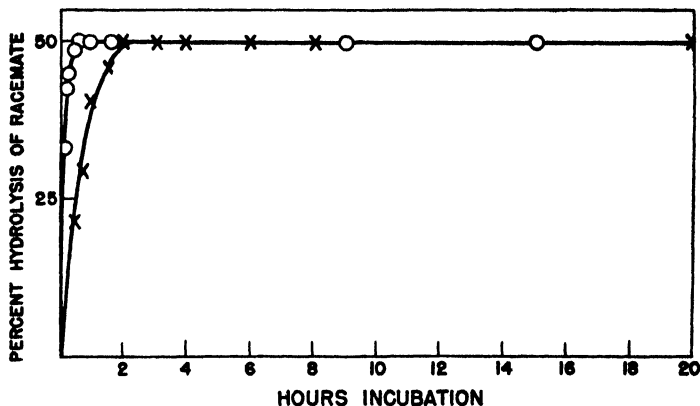
FIG. 1. Asymmetric hydrolysis of chloroacetyl-DL-alanine (X) and of chloroacetyl-DL-phenylalanine (O) by hog kidney preparation and by beef pancreatic carboxypeptidase respectively. Digests consisted of 1 cc. of enzyme solution, 2 cc. of 0.07 M phosphate buffer at pH 7.4, and 1 cc. of either water or 0.05 M substrate solution. Incubation temperature 37°. Hydrolysis followed by ninhydrin-CO_2 analyses.

from hot water with the aid of a little norit, the yields of optically pure L-amino acids were reduced to 27, 28, and 14 gm., respectively (Table I).

The mother liquor from the digestion mixture is combined with the washings and concentrated *in vacuo* to a low bulk. A small amount of L-amino acid which appears during this condensation is ignored. The concentrated solution is layered with ethyl acetate and then treated with chilled, concentrated hydrochloric acid to pH 2.0. The chloroacetyl-D-amino acid is extracted several times into ethyl acetate, the extracts combined and dried for a few minutes over anhydrous sodium sulfate, and finally filtered. The ethyl acetate is removed *in vacuo*, and the oily residue washed twice with petroleum ether to remove chloroacetic acid. After this procedure, the residue crystallizes readily on chilling. The yield of chloroacetyl-D-phenylalanine, tyrosine, or tryptophan is 70 to 80

per cent of the theoretical, based upon the amount of chloroacetyl-DL-amino acid taken.

The melting points and total nitrogen values for chloroacetyl-D-phenylalanine, chloroacetyl-D-tyrosine, and chloroacetyl-D-tryptophan were, respectively, 125° and 5.7 per cent (calculated 5.8), 154° and 5.2 per cent (calculated 5.4), and 157° and 9.9 per cent (calculated 10.0). The melting

TABLE I

Specific Rotations at 23° of Optical Isomers of Racemic Amino Acids Resolved by Asymmetric Enzymatic Hydrolysis of Their N-Chloroacylated Derivatives

Compound	Present data					Data in literature	
	L form		D form		N calcu- lated	L form	D form
	$[\alpha]_D$	N ob- served	$[\alpha]_D$	N ob- served		$[\alpha]_D$	$[\alpha]_D$
Phenylalanine	−34.8*	8.4	+34.8†	8.4	8.5	−35.1 (9)	+35.0 (9), +34.6 (13)
Chloroacetyl- phenylalanine	+50.4‡	5.7	−50.8§	5.7	5.8	+51.8 (9)	
Glycylphenyl- alanine	+41.5‖	12.4	−41.7‖	12.4	12.6	+42.0 (9)	
Tryptophan	−32.2¶	13.4	+32.5**	13.5	13.7	−32.1 (12), −32.2 (14)	+32.5 (12)
Tyrosine	−7.3††	7.7	+7.2‡‡	7.7	7.7	−8.6 (15) −7.3 (14)	+8.6 (15)

* 0.505 gm. in 25 cc. of water.

† 0.502 gm. in 25 cc. of water.

‡ 0.512 gm. in 25 cc. of absolute alcohol.

§ 0.508 gm. in 25 cc. of absolute alcohol.

‖ 0.250 gm. in 25 cc. of water.

¶ 0.120 gm. in 25 cc. of water.

** 0.130 gm. in 25 cc. of water.

†† 1.235 gm. in 25 cc. of 6 N HCl. The specific rotation noted for a recrystallized commercial specimen of L-tyrosine in 6 N HCl was −6.9.

‡‡ 1.289 gm. in 25 cc. of 6 N HCl.

points for the corresponding L forms are 125° (126° corrected (9)), 156° corrected (10), and 159° corrected (11).

Part of the chloroacetyl-D-phenylalanine was aminated with concentrated ammonia to form the corresponding glycyl-D-phenylalanine. A part also of the L-phenylalanine was chloroacetylated to form chloroacetyl-L-phenylalanine (9), and the latter subsequently aminated to form the corresponding glycyl-L-phenylalanine (9). The two isomeric glycylphenylalanines were recrystallized from water. The optical and analytical data for these compounds are given in Table I.

20 gm. of chloroacetyl-D-phenylalanine and 25 gm. of chloroacetyl-D-tyrosine are each suspended in 10 times the amount of 2 N hydrochloric acid and refluxed for 1 hour. After treatment with a little norit, the solutions are evaporated *in vacuo* to dryness, and evaporated twice more with water to remove excess hydrochloric acid. The residue is dissolved in a little cold water and treated with 4 N lithium hydroxide to pH 5.0. The precipitated free D-amino acid is filtered and the mother liquor evaporated *in vacuo* to obtain a second crop of crystals. The combined yields are recrystallized from hot water. The final, dried product of pure D-phenylalanine or D-tyrosine amounted to 8 and 11 gm. respectively.

29 gm. of chloroacetyl-D-tryptophan are suspended in 20 times the amount of 2 N sulfuric acid and refluxed for 2 hours (12). The resulting clear solution, which is pink in color, is diluted with 3 times its volume of distilled water and treated with warm baryta solution to slight excess (about pH 9). Glacial acetic acid is immediately added to lower the pH to 4.8, and the mixture is warmed and filtered through a layer of norit. The colorless, clear filtrate is concentrated *in vacuo* until glistening crystals of D-tryptophan appear. After standing several hours at 5°, the amino acid is filtered by suction, washed with a little cold water, and recrystallized from hot water. The final product is washed with alcohol and ether. The yield of optically pure D-tryptophan is 9 gm.

The yields on chloroacetylation of DL-phenylalanine, tyrosine, and tryptophan amount to about 50 to 60 per cent of the theoretical. Our over-all yields therefore from DL-phenylalanine, tyrosine, and tryptophan amount for the purified L isomers to 30, 30, and 28 per cent, and for the purified D isomers to 25, 24, and 18 per cent of the theoretical, respectively. These figures are based upon the use of 0.5 mole or more of the racemic amino acid both here and as described in previous papers (1, 2). It is quite probable that the yields could be considerably improved by more careful exploitation of the mother liquors at each crystallization step, and by reworking the mother liquors from the chloroacetylation procedures.

The optical and analytical data on the preparations of the various isomers are given in Table I. The resolution of the original racemic forms of the amino acids is clear cut.[1]

SUMMARY

1. Racemic phenylalanine, tyrosine, and tryptophan have been readily resolved into their optical isomers by subjecting their *N*-chloroacetylated

[1] Behrens, Doherty, and Bergmann have reported a preparation of the isomers of DL-phenylalanine by the difference in rates of synthesis by papain of the anilides of acetyl-DL-phenylalanylglycine (16). L-Phenylalanine and D-phenylalanine with specific rotations of −35.6° and +32.3° were obtained. The latter compound may have been contaminated with the L form.

derivatives to asymmetric hydrolysis by a purified beef pancreas carboxypeptidase preparation.

2. In contradistinction to the susceptible chloroacetyl derivatives of phenylalanine and of tryptophan, the corresponding acetyl derivatives are very resistant to the action of pancreatic carboxypeptidase.

BIBLIOGRAPHY

1. Fodor, P. J., Price, V. E., and Greenstein, J. P., *J. Biol. Chem.*, **178,** 503 (1949).
2. Price, V. E., Gilbert, J. B., and Greenstein, J. P., *J. Biol. Chem.*, **179,** 1169 (1949).
3. Anson, M. L., *J. Gen. Physiol.*, **20,** 663 (1937).
4. Grassmann, W., in Handbuch der Biochemie des Menschen und der Tiere, 2nd edition, Jena (1930).
5. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **134,** 225 (1940).
6. Bergmann, M., and Fruton, J. S., *Advances in Enzymol.*, **1,** 63 (1941).
7. Stahmann, M. A., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **164,** 753 (1946).
8. Neurath, H., Elkins, E., and Kaufman, S., *J. Biol. Chem.*, **170,** 221 (1947).
9. Fischer, E., and Schoeller, W., *Ann. Chem.*, **357,** 1 (1907).
10. Fischer, E., *Ber. chem. Ges.*, **37,** 2486 (1904).
11. Abderhalden, E., and Kempe, M., *Ber. chem. Ges.*, **40,** 2737 (1907).
12. Berg, C. P., *J. Biol. Chem.*, **100,** 79 (1933).
13. Ehrlich, F., and Wendel, A., *Biochem. Z.*, **8,** 399, 438 (1908).
14. Dunn, M. S., and Rockland, L. B., *Advances in Protein Chem.*, **3,** 296 (1947).
15. Fischer, E., *Ber. chem. Ges.*, **32,** 3638 (1899).
16. Behrens, O. K., Doherty, D. G., and Bergmann, M., *J. Biol. Chem.*, **136,** 61 (1940).

THE ACONITE ALKALOIDS

XXIII. OXIDATION OF ISOPYROOXODELPHONINE, DIHYDROISOPYROOXODELPHONINE, AND THEIR DESMETHYLANHYDRO DERIVATIVES

By WALTER A. JACOBS and YOSHIO SATO

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

Studies have been in progress to explore the possibilities of the oxidative degradation of delphinine and aconitine and their derivatives, and we wish to record certain results which have been obtained principally with isopyrooxodelphonine and its dihydro derivative (1). On attempting the chromic acid oxidation of the precursors before saponification, *i.e.* isopyrooxodelphinine and octahydroisopyrooxodelphinine, the reagent acted in a different manner and far less readily than after saponification. With dihydroisopyrooxodelphonine, ($C_{24}H_{37}O_7N$), there resulted in good yield the *saturated keto acid*, $C_{24}H_{35}O_8N$, in which the four methoxyl groups remained unaffected. Diazomethane yielded the *methyl ester* which was not obtained crystalline but was readily purified by sublimation. From the ester a crystalline *oxime* was prepared. All of the oxygen atoms are thus accounted for by the four methoxyl groups and the carboxyl, carbonyl, and lactam groups. The same substance was obtained but much less satisfactorily with permanganate in acetone solution.

The experience with the chromic acid oxidation of the unsaturated isopyrooxodelphonine proved more involved. The major direct oxidation product is an *unsaturated keto acid*, $C_{24}H_{33}O_8N$ ($[\alpha]_D^{25} = +48°$). In this substance the oxygen atoms have also been accounted for by four methoxyl groups, the carboxyl and lactam groups, and an apparently less reactive carbonyl group. The acid yielded a crystalline *methyl ester*. Attempts to prepare an *oxime* from the latter encountered difficulties since the reaction occurred less readily than with the above dihydro derivative. Further study of the preparation of the oxime was abandoned in view of the observation that the unsaturated acid was converted by hydrogenation to the above saturated keto acid, $C_{24}H_{35}O_8N$, obtained directly from dihydroisopyrooxodelphonine. The presence of the carbonyl group in the unsaturated acid is therefore certain.

A complication developed in the gradual transformation of the unsaturated acid to a neutral isomer, found to be an apparently *saturated ketolactone*, $C_{24}H_{33}O_8N$ ($[\alpha]_D^{26} = +74°$). This isomerization was facilitated in acid solution or by sublimation of the acid. The lactone was readily

saponified with dilute alkali or ammonia to the original unsaturated acid. All attempts to hydrogenate the lactone were unsuccessful. However, in the presence of ammonia, which caused saponification, hydrogenation to the above dihydroketo acid readily occurred. Since experiments to prepare an oxime were unsuccessful, the reactivity of the carbonyl group assumed to be retained in the ketolactone appears to have become greatly hindered.
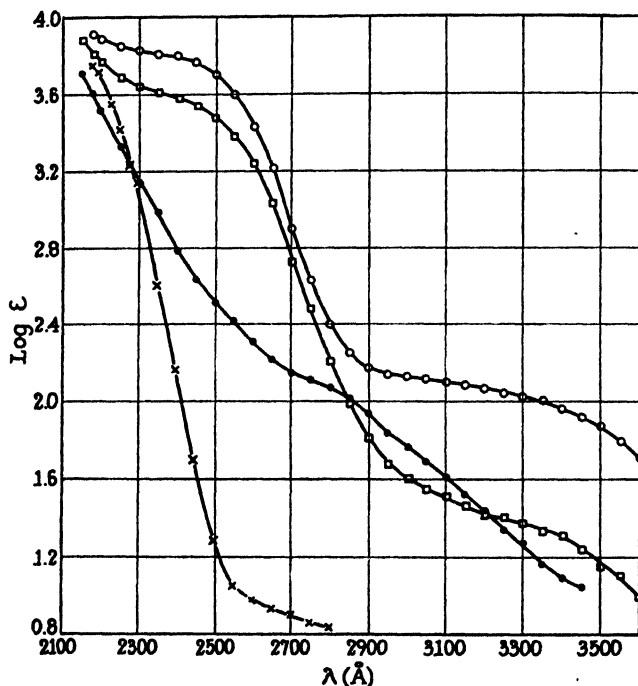
Fig. 1. ×, isopyrooxodelphonine; □, the unsaturated keto acid, $C_{24}H_{33}O_8N$; ○, the unsaturated keto acid methyl ester; ●, the saturated ketolactone, $C_{24}H_{33}O_8N$.

The ultraviolet absorption spectra curves obtained with these substances as shown in Fig. 1 have afforded some suggestions for their structural interpretation. As a possible reference the previously reported curve obtained from isopyrooxodelphonine (2) is repeated here. This shows end-absorption. The curve obtained with the methyl ester of the dihydroketo acid (Fig. 2) shows a band which can be attributed to the carbonyl group. The curves of the unsaturated keto acid and its methyl ester (Fig. 1), although the short wave maxima are low, might be suggestive of a $\Delta^{\alpha, \beta}$-unsaturated ketone. The curve (Fig. 1) of the saturated ketolactone $C_{24}H_{33}O_8N$ does not appear characteristic and the general absorption is such

as to mask carbonyl absorption alone. From the data obtained it is suggested that the unsaturated keto acid is of $\Delta^{\alpha, \beta}$-unsaturated ketonic character (Formula II) and, on isomerization to the saturated lactone, addition of the carboxyl group to the double bond occurs, as presented in the partial Formula III. On saponification of the latter, an unstable intermediate β-hydroxy ketonic structure may be first formed which loses water with the production of the original unsaturated keto acid.
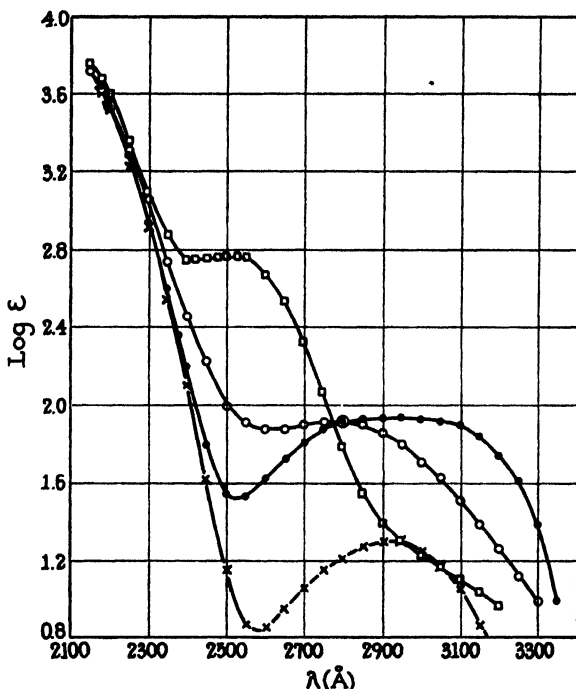
Fig. 2. O, the dihydroketo acid methyl ester; ●, the β-diketone; ×, the β-hydroxyketone; □, the monoketone (?), $C_{21}H_{27}O_6N$; all in ethanol.

The saturated keto acid, $C_{24}H_{35}O_8N$ (Formula IV), on sublimation behaves differently. It also yields a neutral substance but this involves loss of a mole of water. Analysis of the product indicated the formulation $C_{24}H_{33}O_7N$. The substance was found to be readily cleaved by alkali, not only with the regeneration of the original keto acid but with the formation of an apparently isomeric substance. A first conclusion which attributed the loss of water to lactonization on the enolized carbonyl group with formation of an unsaturated lactone was soon abandoned in favor of the interpretation that the condensation had caused the production of a β-diketone $C_{24}H_{33}O_7N$ (Formula V) of unusual lability towards alkali. On

hydrogenation of this substance in methanol, 1 mole of H_2 was absorbed with the production of a substance $C_{24}H_{35}O_7N$ (Formula VI). Contrary to its precursor, the latter did not consume alkali on attempted saponification. It has been provisionally interpreted as a *β-hydroxyketone* formed by hydrogenation of one CO group.[1] When the hydrogenation was performed in acetic acid, only a relatively small amount of the hydroxy ketone was obtained. The major product, as shown by the analytical data, resulted from a further loss of an oxygen atom. It appears possible that the hydrogenation proceeds in a different manner in acid solution with the formation of a *monoketone*, $C_{24}H_{37}O_6N$. The ultraviolet absorption spectra ob-

tained with these substances are shown in Fig. 2. The curve obtained with the so called monoketone, $C_{24}H_{37}O_6N$, is particularly unsatisfactory and this substance requires further investigation. The reason for the somewhat abnormal curves obtained with the β-diketonic derivative and its β-hydroxy ketonic hydrogenation product will also require explanation. The same curves were practically duplicated in later experiments.
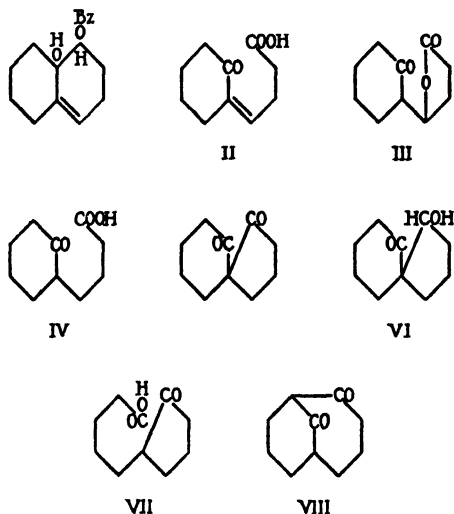
On the basis of these observations certain tentative conclusions may be summarized regarding the structural features of the alkaloid involved. Since oxidation of isopyrooxodelphinine and its hydrogenation product occurs readily only after saponification to yield keto acids, it is suggested

---

[1] Since the hydrogenation of the unsaturated keto acid stops at the saturated keto acid stage (Formula IV), it would appear that its CO group is relatively resistant to hydrogenation. It seems therefore that the new CO group is the one reduced as shown in Formula VI.

that the original benzoyl group protects a secondary hydroxyl which adjoins a tertiary hydroxyl group, as indicated in partial Formula I. In the unsaturated keto acid $C_{24}H_{33}O_8N$ from isopyrooxodelphonine, the double bond of the latter is assumed to be in the position or has shifted to that shown in Formula II, α, β to the CO group. The carboxyl group would be properly oriented in regard to the $\Delta^{\alpha,\beta}$-carbonyl grouping to permit addition to the double bond with formation of the isomeric saturated ketolactone, as shown in Formula III.[2] The latter when saponified could pass through the unstable β-hydroxyketo acid stage which loses water to form again the original unsaturated keto acid (Formula II). The latter in turn on hydrogenation yields the saturated keto acid (Formula IV) obtained directly by oxidation of dihydroisopyrooxodelphonine. It is assumed that loss of water from the latter on pyrolysis causes cyclization to form the β-diketone of spirane structure as presented in Formula V, which can in turn be hydrogenated to the β-hydroxy ketone (Formula VI).[1] The different ways in which such a diketone could be cleaved with alkali could explain the observations made. The point of rupture would determine either regeneration of the original keto acid or formation of an isomeric keto acid, as shown in Formula VII. The formation of a diketone as presented in Formula VIII has also been considered, since it might permit the other transformations observed, but it appears less likely. In all of these reactions the four methoxyl groups remain unaffected and there is no evidence that the lactam group participates in any of the transformations.

The apparent greater resistance to demethylation of the dihydroisopyrooxodelphinine series as discussed in the previous communication (2) has been substantiated by a study of the action of zinc chloride and HCl on the saturated keto acid, $C_{24}H_{35}O_8N$ above. Only two methyl groups and 1 mole of water were readily removed under the conditions used with the formation of the *dimethylanhydroketo acid* $C_{22}H_{29}O_7N$. The loss of water occurs presumably with formation of an oxidic ring and is restricted here to the hydroxyl groups exposed by the dimethylation, since they are the only ones available.

In the previous communication the oxidation of desmethylanhydroisopyrooxodelphinine was shown to yield a monoketo derivative. Following the above experience with isopyrooxodelphonine, it was of interest to study the oxidation of the saponification product of the former, desmethylanhydroisopyrooxodelphonine. The result appeared to parallel that obtained

---

[2] An alternative but less favored interpretation would place the conjugated double bond in the first ring. The lactone which is formed would then be a less likely ε-lactone or a δ-lactone if the carboxyl group arises from cleavage of a cyclopentane ring. In the latter case appropriate changes would be required in the other formulas given.

with isopyrooxodelphonine, but with the additional oxidation of the secondary hydroxyl group exposed by the demethylation. After long continued extraction of the diluted reaction mixture with chloroform, two isomeric substances were obtained, one an acid and the other of neutral character. Analysis of these indicated the formulation $C_{20}H_{21}O_7N$. The acid has been interpreted as the *unsaturated desmethylanhydrodiketo acid* in analogy with the experience with the above tetramethoxy derivative. This is assumed to be the initial product which gradually and incompletely lactonizes on the double bond to the isomeric neutral *saturated desmethylanhydrodiketolactone*. The latter is readily saponified and, although gradually attacked by dilute ammonia or Na_2CO_3, it dissolves readily in fixed alkali. If these substances have been correctly interpreted, the original methoxyl groups after demethylation to hydroxyls are sufficiently removed from the assumed vicinal tertiary and secondary hydroxyl groups not to participate in the oxidative cleavage of the latter to CO and COOH.

In a preliminary study of the oxidation of the dihydro derivative, desmethylanhydrodihydroisopyrooxodelphonine, the expected *desmethylanhydrodihydrodiketo acid*, $C_{20}H_{33}O_7N$, was obtained. This product also required long continued extraction for removal from the diluted oxidation mixture.

It was of interest in this connection to attempt to limit the factors which determine the isomerism between the pyro-α-oxodelphinine and the isopyrooxodelphinine series. For this purpose the previously described octahydropyro-α-oxodelphinine ($[\alpha]_D$) $= -14°$) (1) was saponified to *dihydropyro-α-oxodelphonine*. The latter was not obtained crystalline but its oxidation with chromic acid paralleled that described above with dihydroisopyrooxodelphonine. A *dihydroketo acid*, $C_{24}H_{35}O_8N$, resulted and its melting point (131–136°) was lower than that of the above dihydroketo acid (149–154°). Although the substances from each source thus appeared to be different, the resemblance in the other properties studied was sufficiently close to render a conclusion in this respect difficult.

<center>EXPERIMENTAL</center>

Isomeric Unsaturated Keto Acid and Saturated Ketolactone—1.1 gm. of isopyrooxodelphonine (1) dissolved in 35 cc. of acetic acid were gradually treated with 5 cc. of Kiliani chromic acid solution (53 gm. of CrO_3 and 80 gm. of H_2SO_4 in 400 cc. of H_2O). Within 10 minutes at room temperature the reagent was used up. The diluted mixture was treated with an excess of sodium acetate solution and then exhaustively extracted with chloroform. The washed extract was reextracted with an excess of dilute Na_2CO_3 followed by water. The aqueous extract after reacidification with

H_2SO_4 was reextracted exhaustively with chloroform. On concentration and drying *in vacuo* 0.7 gm. of resin resulted.

In preliminary experiments it was found that such material on standing, especially in solution, very gradually yielded crystalline neutral material. After several months the main resin had partly crystallized and, when treated with a small volume of methanol, became copious. The crystals were collected with methanol followed by ether and amounted to 0.127 gm. The substance was neutral and formed small stout tetrahedral or hexagonal prisms which melted at 234–235°. In dilute methanol solution with nitroprusside and either Na_2CO_3 or NaOH, a negative yellow color quickly developed.

$$[\alpha]_D^{25} = +74° \ (c = 0.45 \text{ in } 95\% \text{ ethanol})$$

$C_{24}H_{33}O_5N$.　Calculated.　C 62.17, H 7.18, OCH_3 26.79
　　　　　Found.　(a) " 62.05, " 7.22,
　　　　　　　　(b) " 62.63, " 7.09 　 " 　26.40
　　　　　　　　(c) " 62.30, " 7.15

Although the substance was not directly soluble in dilute Na_2CO_3 or ammonia, it gradually dissolved in dilute NaOH, due to saponification.

8.96 mg. of substance suspended in 1 cc. of ethanol on direct titration against phenolphthalein slowly but progressively consumed 0.203 cc. of 0.1 N NaOH; calculated for 1 equivalent, 0.193 cc. On addition of excess reagent and refluxing for $2\frac{1}{2}$ hours, no further consumption was noted.

2.115 mg. of substance were refluxed in 0.1 cc. of ethanol and 0.1 cc. of 0.1 N NaOH for $2\frac{1}{2}$ hours and titrated. Found, 0.0475 cc.; calculated for 1 equivalent, 0.0456 cc.

On acidification of the titration solution to Congo red with dilute HCl, the mixture gradually crystallized on standing.

Found, C 62.38, H 7.25

The mother liquor from the above 0.127 gm. of lactone was concentrated to small volume and diluted with H_2O. A resinous suspension formed which, when warmed and seeded with the lactone, gradually crystallized. However, after several days aggregates of much larger crystals appeared. 0.17 gm. of this material was removed mechanically from the suspension of smaller crystals and proved to be the acid now to be described.

The remaining suspension of smaller crystals was carefully treated with ammonia as long as apparent solution occurred, and sufficient to be in slight excess. The undissolved fraction which was collected with water amounted to 48 mg. and proved to be the neutral lactone.

The filtrate after acidification with acetic acid and seeding gradually yielded compact pointed micro prisms. 40 mg. were collected with water. The substance was readily soluble in dilute Na_2CO_3 and ammonia and

under the microscope did not show a sharp melting point, apparently owing to partial transformation into the neutral lactone. About 30 per cent melted at 196–199°, about 50 per cent at 230–233°, and the remainder at 241–245°.

$$[\alpha_D^{25}] = +48° \ (c = 0.415 \text{ in } 95\% \text{ ethanol})$$

For analysis it was dried at 110° and 0.2 mm.

$C_{24}H_{33}O_8N$. Calculated, C 62.17, H 7.18; found, C 61.84, H 6.93

As in the case of the lactone, the acid showed no Legal reaction with nitroprusside.

30 mg. of the above acid were heated gradually in a sublimation apparatus at 0.03 mm. When the bath temperature reached 210°, most of the substance appeared to melt and at about 225° a deposit appeared on the condenser. During 15 minutes the bath was gradually raised to 275°. The sublimate was washed off with acetone and, when dried, weighed 26 mg. This crystallized readily in a small volume of methanol. This fraction weighed 9 mg. and was followed by an additional 5 mg. The substance was not soluble in dilute ammonia or Na_2CO_3.

$$[\alpha]_D^{26} = +74° \ (c = 0.42 \text{ in } 95\% \text{ ethanol})$$

In other respects it proved identical with the above neutral lactone.

The above aqueous mother liquor from the acid fraction which had been acidified with acetic acid was now further acidified to Congo red with H_2SO_4. On long standing a final fraction of 35 mg. of neutral lactone was obtained which melted at 228–231°.

Methyl Ester—The previous acid (70 mg.) was treated with excess diazomethane in acetone. The concentrated solution crystallized as micro leaflets or rods. When crystallized from a small volume of cold ether, 52 mg. were obtained which gradually melted under the microscope at 197–202° with crystals remaining until 205°.

$C_{25}H_{35}O_8N$. Calculated, C 62.86, H 7.39; found, C 62.68, H 7.21

For the preparation of the *oxime* 20 mg. of ester were gently warmed to dissolve with a mixture of 12 mg. of hydroxylamine hydrochloride and 30 mg. of sodium acetate in 2 cc. of methanol. After several days, the concentrated mixture on careful dilution yielded 5 mg. of a crystalline fraction which contained unchanged starting material. The concentrated mother liquor on further manipulation yielded 7 mg. of a microcrystalline fraction which melted under the microscope at 173–178°. A small amount apparently of starting material persisted till 192–196°.

$C_{25}H_{36}O_8N_2$. Calculated, N 5.69; found, N 5.04

Dihydroketo 'Acid, $C_{24}H_{35}O_8N$—0.3 gm. of dihydroisopyrooxodelphonine in 10 cc. of acetic acid was gradually treated at room temperature with Kiliani chromic acid solution. Following the addition of 0.9 cc. or about 2 equivalents of O, the reaction appeared more gradual. When a total of 1.2 cc. had been consumed, the mixture was diluted and thoroughly extracted with chloroform. The extract after washing with a small amount of water was concentrated finally *in vacuo* to remove excess acetic acid. The residue was redissolved in chloroform and the solution then reextracted with dilute Na_2CO_3. The latter was reacidified with H_2SO_4 and, in turn, thoroughly extracted with chloroform. The dried extract after concentration yielded 0.22 gm. of residue. The latter was dissolved in a small volume of methanol and after dilution gradually crystallized with about 1 mole of H_2O as stout nearly rhombic or rectangular prisms or platelets followed by some pointed blades. The acid melted gradually from 149–154° after preliminary sintering, especially above 140°. The melting point, however, varied considerably with the conditions of crystallization. From benzene it formed needles with or without solvent which sintered above 150° and gradually melted above 156°; but crystals persisted to 166°. It is sparingly soluble in H_2O and cold benzene, but readily soluble when warmed. It is soluble in alcohol and acetone.

$$[\alpha]_D^{25} = +7° \ (c = 0.98 \text{ in } 50\% \text{ ethanol})$$

For analysis it was dried at 110° and 0.2 mm.

$C_{24}H_{35}O_8N \cdot H_2O$. Calculated, H_2O 3.73; found, H_2O 3.73
$C_{24}H_{35}O_8N$. Calculated. C 61.90, H 7.58, OCH_3 26.67
Found. " 62.02, " 7.46, " 26.17

8.578 mg. of substance on titration with 0.1 N NaOH against phenolphthalein required 0.188 cc. Calculated for 1 equivalent, 0.185 cc. When boiled with excess reagent further consumption was negligible.

The oxidation with permanganate was much less satisfactory. 50 mg. of the dihydro derivative in 5 cc. of acetone were treated with 40 mg. of $KMnO_4$. Since there was no apparent reaction after 15 minutes, 0.05 cc. of acetic acid was added. Only a very gradual reaction occurred at room temperature and the reagent persisted for 2 days. The filtrate from MnO_2 was diluted and concentrated to remove acetone and the mixture was then made alkaline with dilute Na_2CO_3 and extracted with chloroform. The latter yielded 22 mg. of neutral residue which partly crystallized from acetone-ether when seeded with starting material.

The above Na_2CO_3 extract was acidified with H_2SO_4 and, after extraction with chloroform, yielded 31 mg. of a resin. This was dissolved in a drop of methanol followed by dilution. When seeded, crystallization

occurred and 12 mg. of substance were collected which proved identical with the above acid.

Found, C 61.93, II 7.54

Methyl Ester—75 mg. of the previous acid in acetone solution were treated with excess diazomethane. Since attempts to crystallize the ester were unsuccessful, the resin obtained after removal of solvent was sublimed at 0.05 mm. pressure from a bath heated from 200–230°. A colorless resin was collected.

$C_{25}H_{37}O_8N$. Calculated, C 62.59, H 7.78; found, C 62.57, H 7.75

1.863 mg. of substance were heated at 100° for $2\frac{1}{2}$ hours in 0.1 cc. of ethanol and 0.1 cc. of 0.1 N NaOH and titrated against phenolphthalein. Found, 0.0422 cc. Calculated for 1 equivalent, 0.0389 cc.

Oxime of Methyl Ester—20 mg. of the ester in methanol were heated for 1 hour with a mixture of hydroxylamine hydrochloride and sodium acetate. The concentrated mixture on dilution yielded 14 mg. of crystals. On progressive dilution of its concentrated methanol solution, the oxime crystallized as glistening 4-sided micro prisms or platelets which melted at 140–142°.

$C_{25}H_{38}O_8N_2$. Calculated. C 60.69, H 7.75, N 5.67
Found.   " 60.87, " 7.74, " 5.95

The relationship of the oxidation products from isopyrooxodelphonine and its dihydro derivative was shown in two ways.

0.275 gm. of isopyrooxodelphonine was oxidized with Kiliani solution as described above, and the chloroform extract was in turn extracted with dilute Na_2CO_3. The latter after acidification was reextracted with chloroform which yielded 0.15 gm. of a resinous acid product. The latter in methanol solution was hydrogenated with 50 mg. of platinum oxide. Although the H_2 absorption appeared complete in 2 hours, the operation was continued for 2 days. The apparent absorption in excess of the catalyst requirement was about 9 cc. This was in excess of the theory and possibly due to side reactions. The reaction mixture was diluted with chloroform, filtered, and the filtrate extracted with dilute Na_2CO_3. The chloroform phase was now found to contain an appreciable neutral fraction, since on concentration it yielded 89 mg. of residue. When treated with methanol, characteristic crystals of the neutral lactone separated. This fraction yielded 21 mg. and more separated from the mother liquor. It melted at 232.5–236.5'.

$C_{24}H_{33}O_4N$. Calculated, C 62.17, H 7.18; found, C 62.30, H 7.17

Due to lactonization this fraction therefore escaped hydrogenation.

The above Na_2CO_3 extract after reacidification with H_2SO_4 was reextracted with chloroform. The latter yielded 62 mg. of a resin. The solution in a few drops of methanol, when carefully diluted, gave successive fractions of the dihydroketo acid as microtriangular leaflets which melted at 152.5°.

For analysis it was dried at 110° and 0.2 mm.

$C_{24}H_{35}O_8N$. Calculated, C 61.90, H 7.58; found, C 62.04, H 7.53

In another experiment 48 mg. of the unsaturated ketolactone, $C_{24}H_{33}O_8N$, were shaken in 95 per cent ethanol with 50 mg. of platinum oxide and H_2. After absorption of 12 cc. by the catalyst, no further absorption occurred even on heating to dissolve the sparingly soluble substance. The apparatus was then exhausted of H_2 and disconnected. A few drops of ammonia in sufficient excess were added; the apparatus was refilled with H_2 and the operation was continued. The crystals gradually dissolved, due to saponification, and H_2 absorption also became apparent. After 50 minutes the absorption (3.4 cc.) was somewhat in excess of 1 mole (about 2.5 cc.) and had practically stopped. The filtrate from the catalyst, after repeated concentration *in vacuo* to dryness, gave a residue of the free acid which, when dissolved in warm water and seeded, readily crystallized as micro prisms or leaflets which melted at 150–155° after preliminary sintering.

For analysis it was dried at 110° and 0.2 mm.

Found, C 61.71, H 7.59

β-Diketo Derivative—0.4 gm. of the dihydroketo acid was gradually heated in a sublimation apparatus with the pressure at about 0.01 to 0.05 mm. With the bath at 190° slight sublimation became apparent and the temperature was gradually raised during 10 minutes to 205° and then to 235°, when sublimation was practically completed. Following a final period the temperature reached 245°. The resinous deposit was dissolved in chloroform and the latter was extracted first with dilute Na_2CO_3 and then with water. The dried solution on concentration yielded 0.32 gm. of a neutral resin. The latter, when dissolved in acetone followed by addition of ether, crystallized as spear-headed plates or leaflets. 0.175 gm. was collected with ether. It melted at 141–142° after slight preliminary sintering.

$C_{24}H_{33}O_7N$. Calculated, C 64.39, H 7.44; found, C 64.34, H 7.40

In methanol or pyridine solution with sodium nitroprusside and dilute Na_2CO_3 it gave a red color, whereas the original keto acid showed a much more slowly developing reaction. With dilute NaOH there was less contrast and in both cases a deep red color developed.

The substance was readily cleaved with dilute alkali. 1.965 mg. of sub-

stance in 0.1 cc. of ethanol were titrated against phenolphthalein at room temperature with 0.1 N NaOH. It consumed the alkali gradually up to 0.0358 cc. Calculated for 1 mole, 0.0439 cc.

The ultraviolet absorption spectrum is recorded in Fig. 2.

As a check on its relation to the original keto acid, its alkali cleavage was studied as follows: 35 mg. of the substance were suspended in 1 cc. of H_2O and treated with excess ammonia. On stirring, and especially when warmed, it gradually dissolved. A small amount of ethanol was added with some additional ammonia and the mixture was repeatedly boiled down after H_2O addition to remove all ethanol and ammonia. When seeded with the keto acid, crystallization occurred. 5 mg. were collected which melted at 149–151° and were soluble in dilute ammonia. Manipulation of the mother liquor yielded about 1 mg. more. From benzene with ether it formed needles which sintered above 150° and gradually melted at 155–159° but not completely till 165°.

$$[\alpha]_D^{25} = +7.5° \ (c = 0.39 \text{ in } 50\% \text{ ethanol})$$
Found, C 61.58, H 7.66

The major portion remained in the mother liquor. Because of initial crystallization difficulties this was repeatedly extracted with chloroform and the concentrated solution was reextracted with dilute Na_2CO_3, followed by H_2O. Only a negligible fraction remained in the chloroform phase. The Na_2CO_3 extract was acidified with H_2SO_4 and repeatedly extracted with chloroform. The latter yielded 26 mg. of a resin. When dissolved in benzene and seeded with the keto acid, a mg. or so of the above substance crystallized. The mother liquor was concentrated to dryness. The residue dissolved in a little acetone followed by ether crystallized on standing as rosettes of flat needles or leaflets. 7 mg. were collected. It lost birefringence under the microscope about 75–80° and was definitely different from its precursor and probably isomeric. Its properties and the amount available made necessary a postponement of its further detailed study. For analysis it was dried at 100° and 0.2 mm.

$C_{24}H_{35}O_4N$. Calculated, C 61.90, H 7.58; found, C 61.30, H 7.45

β-Hydroxyketo Derivative—50 mg. of the above β-diketo derivative, when hydrogenated in methanol with 30 mg. of platinum oxide, absorbed slightly more than 1 mole of H_2 beyond the catalyst requirements. This occurred within an hour but the operation was continued for several hours. The concentrated filtrate yielded a resin which from a few drops of acetone on careful ether addition crystallized mostly as microtriangular prisms or platelets. The yield in this fraction was about 20 mg. A small additional amount was obtained from the mother liquor but much remained as a noncrystalline resin. This could not be crystallized when seeded with the

substance described below. After recrystallization from acetone-ether, the product melted at 212–213°.

$C_{24}H_{35}O_7N$. Calculated, C 64.10, H 7.85; found, C 64.07, H 7.78

When refluxed for several hours with a mixture of equal parts of methanol and 0.1 N NaOH, no consumption of alkali was noted.

The ultraviolet absorption spectrum is recorded in Fig. 2.

70 mg. of the diketone were hydrogenated with 50 mg. of platinum oxide in acetic acid. Although most H_2 absorption occurred during the 1st few hours, the operation was continued overnight when the apparent absorption beyond the catalyst was 10 to 11 cc. or a possible 3 moles. Chloroform was used during filtration from the catalyst and the filtrate was concentrated in vacuo to dryness. A solution of the residue in chloroform, when extracted with dilute Na_2CO_3, yielded negligible acidic material. The neutral fraction was obtained as a resin on removal of the chloroform and yielded from a concentrated acetone-ether solution a minor initial fraction. When collected with ether, 4 mg. were obtained. This together with a fraction from a second experiment was recrystallized from acetone-ether. It separated as small microtriangular or hexagonal prisms which agreed in properties with the above substance.

Found, C 64.13, H 8.06

The major product in the mother liquor was obtained in several fractions from ether in a yield of 40 to 50 mg. It formed micro platelets which melted at 168–170°. It gave practically no Legal test and absorbed no alkali on direct titration or after heating several hours with 0.1 N NaOH.

$C_{24}H_{37}O_6N$. Calculated. C 66.16, H 8.57, OCH_3 28.51
$C_{23}H_{35}O_6N$.   "   " 65.51, " 8.37, " 29.44
Found.   (a) " 65.56, " 8.46, " 28.50
        (b) " 66.04, " 8.57
        (c) " 66.01, " 8.58
        (d) " 65.49, " 8.20

The ultraviolet absorption spectrum obtained is shown in Fig. 2.

Dimethylanhydroketo Acid—50 mg. of the dihydroketo acid, $C_{24}H_{35}O_6N$, were gradually dissolved in a solution of 2 gm. of zinc chloride in 0.7 cc. of 5 per cent HCl at 40°. After 45 minutes the clear diluted solution was extracted continuously with chloroform for several hours. The extracted resin crystallized readily from methanol as small hexagonal micro platelets or larger needles which melted at 272–277° after preliminary sintering.

For analysis it was dried at 110° and 0.2 mm.

$C_{23}H_{29}O_7N$. Calculated. C 62.97, H 6.97, $2(OCH_3)$ 14.80
Found.   (a) " 62.72, " 6.93, " 15.60
        (b) " 62.18, " 6.81, " 15.10
        (c) " 63.42, " 7.14, " 15.41

2.012 mg. of dried substance on direct titration with 0.1 N NaOH against phenolphthalein required 0.0468 cc. Calculated for 1 equivalent, 0.048 cc.

Oxidation of Dihydropyro-α-oxodelphonine—Since dihydropyro-α-oxodelphonine has not been crystallized, the amorphous material obtained by saponification of octahydropyro-α-oxodelphinine (1) was used for the oxidation. For this purpose a solution of 0.1 gm. of the latter in 2.5 cc. of methanol was treated with 1.2 cc. of 10 per cent NaOH. The clear solution after several hours at room temperature was treated with saturated NaCl solution and repeatedly extracted with chloroform. The extract was washed with NaCl solution, dried, and concentrated *in vacuo* to a resin. The latter in a mixture of 3 cc. of acetic acid and 0.3 cc. of H_2O was treated with 0.5 cc. of Kiliani solution. After 45 minutes at room temperature, the diluted mixture was extracted with chloroform in a continuous extractor. The extracted material after drying *in vacuo* weighed 80 mg. A solution in a few drops of methanol followed by H_2O addition gradually yielded a crust of crystals. 29 mg. were collected with H_2O. For recrystallization it was dissolved in H_2O with a slight excess of ammonia, cleared with norit, and the filtrate acidified with acetic acid. It crystallized as 4- or 5-sided boat-shaped or often triangular micro leaflets which melted at 131–136° after preliminary sintering. The mixture with the dihydroketo acid described above melted at an intermediate point, 143–147°.

$$[\alpha]_D^{25} = +2° \ (c = 0.79 \ \text{in} \ 50\% \ \text{ethanol})$$

$C_{24}H_{35}O_5N \cdot H_2O$.   Calculated, H_2O 3.73; found, H_2O 3.32, 3.03

$C_{24}H_{35}O_5N$.   Calculated.   C 61.90, H 7.58
Found.   (*a*) " 62.06, " 7.84
(*b*) " 62.00, " 7.61

Isomeric Unsaturated Desmethylanhydrodiketo Acid and Saturated Diketo Lactone—0.15 gm. of desmethylanhydroisopyrooxodelphonine was dissolved in a mixture of 0.75 cc. of H_2O and 2.25 cc. of acetic acid and gradually treated with 0.96 cc. of Kiliani solution. The reaction became more gradual after 5 minutes. After 25 minutes the diluted solution was treated with 0.7 cc. of saturated sodium acetate and the mixture was continuously extracted with chloroform for 18 hours. 31 mg. of a resin were extracted. After 24 hours more, a second fraction of 31 mg. followed which was succeeded in turn after 48 hours by 32 mg. A final 72 hours yielded 12 mg. The combined fractions in a small volume of water gradually crystallized, especially when crystals were once obtained. This was collected with a small volume of H_2O and weighed 51 mg. It was found to be a mixture of apparently neutral and acid material. For separation the suspension in a little water was carefully treated with ammonia

in slight excess and after thorough mixing the undissolved neutral lactone was collected with a little water. This material which amounted to 16 mg. was not directly soluble in dilute ammonia or Na_2CO_3, but rather quickly in dilute NaOH. It melted gradually over a range of 298–312° after some preliminary sintering about 255–257° and contained solvent.

For analysis it was dried at 110° and 0.2 mm.

$C_{20}H_{21}O_7N$. Calculated, C 61.99, H 5.47; found, C 62.00, H 5.64

For recrystallization this material with a similar fraction from another experiment was dissolved in acetone-H_2O (5:1) and concentrated to remove most of the acetone. The solution slowly crystallized as needles. The substance after collection with water was still not directly soluble in ammonia or dilute Na_2CO_3. It partly softened apparently in solvent at 243° and finally melted at 313–316°.

Found, C 61.53, H 5.56

2.045 mg. of dried substance suspended in 0.1 cc. of ethanol and 0.1 cc. of H_2O were titrated directly with 0.1 N NaOH against phenolphthalein. The alkali consumption was gradual and after complete solution of substance amounted to 0.0524 cc. Calculated for 1 equivalent, 0.0528 cc. When heated for 2 hours with excess alkali, no further consumption occurred.

The ammoniacal filtrate from the neutral fraction was acidified with acetic acid and, when rubbed, the acid gradually crystallized as micro platelets which were mostly rhombic or derived therefrom. 21 mg. were collected with water. It was readily soluble in dilute ammonia and Na_2CO_3 and melted at 301–304°. It contained solvent. For analysis it was dried at 110° and 0.2 mm.

$C_{20}H_{21}O_7N$. Calculated. C 61.99, H 5.47
Found. (a) " 62.00, " 5.38
(b) " 62.10, " 5.49

A suspension of 1.97 mg. of dried substance in 0.2 cc. of H_2O and 0.2 cc. of ethanol was titrated directly with 0.1 N NaOH against phenolphthalein. Found, 0.049 cc.; calculated for 1 equivalent, 0.051 cc. When heated with excess alkali for 2 hours, no further consumption occurred.

Desmethylanhydrodihydrodiketo Acid, $C_{20}H_{22}O_7N$—The following account records a preliminary study in which the desmethylanhydrodihydroisopyrooxodelphonine used was the total resinous product obtained on hydrogenation of desmethylanhydroisopyrooxodelphonine (2) and before crystalline material had become available. It was possibly a mixture of isomers. 0.2 gm. dissolved in 1 cc. of H_2O and 3 cc. of acetic acid was gradually treated with Kiliani solution which was consumed within ½ hour. Con-

tinuous extraction of the diluted mixture with chloroform required an unusual time for completion. The approximate amounts extracted were 30 mg. after 22 hours, followed in succession by 25 mg. after 24 hours, 30 mg. after 24 hours, and 36 mg. after a final 72 hours. A diluted acetone solution of the second fraction on slow evaporation crystallized when nearly dry and the other fractions could then be seeded. The last three fractions were combined by solution in H_2O-acetone (1:9), filtered, and then concentrated repeatedly with dry acetone to remove most of the water. It slowly separated as a microcrystalline powder which was aided by addition of ether. After long standing this was collected with moist acetone in which it was appreciably soluble. It was readily soluble in H_2O and melted at 222–223°. For analysis the substance was dried at 110° and 0.2 mm.

$C_{20}H_{23}O_7N$ Calculated, C 61.67, H 5.96; found, C 61.27, H 5.99

All the quantitative data have been obtained by Mr. D. Rigakos of this laboratory.

SUMMARY

Oxidation of dihydroisopyrooxodelphonine with chromic acid yields a keto acid, $C_{24}H_{35}O_8N$. The unsaturated isopyrooxodelphonine is oxidized to an unsaturated keto acid, $C_{24}H_{33}O_8N$, which can be hydrogenated to the previous acid. Contrary to the latter, the unsaturated keto acid isomerizes with acid or on distillation to a saturated ketolactone, $C_{24}H_{33}O_8N$, which cannot be hydrogenated except when again saponified. The production of the keto acid is interpreted as due to cleavage of vicinal tertiary and secondary hydroxyl groups. The latter occurs as the benzoyl ester in the parent alkaloid. The saturated ketolactone on distillation yields a β-diketo derivative, $C_{24}H_{33}O_7N$, the hydrogenation of which has been studied. The results of the oxidation of the demethylated anhydro derivatives are also described.

The structures involved in these reactions have been discussed and the conclusions drawn.

BIBLIOGRAPHY

1. Jacobs, W. A., and Huebner, C. F., *J. Biol. Chem.*, **170**, 209 (1947).
2. Jacobs, W. A., and Sato, Y., *J. Biol. Chem.*, **180**, 133 (1949).

THE ACTION OF AN ANALOGUE OF ETHANOLAMINE (DIETHANOLAMINE) ON THE FORMATION OF LIVER PHOSPHOLIPIDES*

By CAMILLO ARTOM, W. E. CORNATZER, AND MARIETTA CROWDER

(*From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina*)

In previous investigations (1, 2) a number of substances with a constitution related to that of the main nitrogenous components of tissue phospholipides, choline and ethanolamine, were tested for their ability to stimulate lipide phosphorylation in the liver. Among the compounds tested, diethanolamine caused increases in the phospholipide formation of a degree comparable to, or greater than, the increases observed after the administration of the natural analogue. In such experiments the substances were given in a single large dose and the liver was analyzed a few hours later. It became of interest to study the effects on the rate of lipide phosphorylation and on the composition of liver phospholipides which result from feeding rats over a prolonged period of time with diethanolamine-containing diets.

EXPERIMENTAL

Male albino rats, raised on a stock diet to 100 to 110 gm. of body weight, were transferred to experimental diets in which a mixture of B vitamins (3) was incorporated daily. Two basic diets, both with a low protein content (5 per cent casein), but differing in the level of fat (5 per cent in Diet 26 (4), 32 per cent in Diet 28 (2)) were employed. After 7 or 12 days on the experimental diets, the rats were injected intraperitoneally with a solution of Na_2HPO_4 containing radioactive phosphorus (2 to 4 μc.), and 6 hours later they were killed by decapitation. The lipides were extracted from the liver with alcohol and alcohol-ether and purified with chloroform (5). On aliquots of the chloroform solution the weight of the lipides, their radioactivity (5), and phosphorus content (6) were determined. Additional analyses were carried out on the remaining portions of these extracts, as described below.

Several series of experiments were made at various times of the year, on rats of two different strains (Sherman or Wistar) and under different conditions (diet and time on diet). These differences presumably account for a certain degree of variability in the results obtained on the control groups of the various series. However, in each series the experiments were run simultaneously on animals of the same source and under identical conditions, so that the results from the groups of the same series are directly comparable. In the experiments of Series I the effects of single doses of ethanolamine and diethanolamine were reinvestigated. After 7 days on Diet 26 the rats received 1 cc. of either water, ethanolamine (5 per cent), or diethanolamine (10 per cent) by stomach tube. 5 minutes later, all animals were injected with isotopic phosphate and killed after 6 hours. In Series II to VI, one group of animals was maintained on the unsupplemented diet and one (or more) group on the same diet supplemented with diethanolamine (0.5 per cent). In most of these groups, 5 minutes before the injection of P^{32}, the rats received one additional dose of diethanolamine (100 mg. in 1 cc. of water) by stomach tube, while the animals of the control groups received 1 cc. of water. Series V and VI included also one group each of rats which were maintained on Diet 28 supplemented with ethanolamine (0.5 per cent).

Results

Action of Diethanolamine on Formation of Total Phospholipides (Table I)—After administration of a single massive dose of either ethanolamine or diethanolamine (Series I), the isotopic values increased considerably, as in our previous experiments (1, 2), but, in marked contrast, when diethanolamine was added to the diets for 7 or 12 days (Series II to VI), a decrease in the specific activity occurred, whether or not a massive dose was superimposed on the dietary régime. In two experiments (Series IV and VI) the decrease was a little larger when the massive dose was omitted. On the ethanolamine-containing diets, the specific activity showed a tendency to increase rather than to decrease.

The weight of the liver (calculated for a 100 gm. rat) was increased in the experiments in which diethanolamine was added to Diet 26, but not when the substance was added to Diet 28. It should be pointed out that the rats of the latter groups consumed progressively less food, so that in the last days very little diethanolamine was actually ingested.

Action of Diethanolamine on Formation of Choline-Containing and Non-Choline-Containing Phospholipides—After evaporation of the chloroform from the pooled extracts, the lipides were dissolved in cold methanol. Visible amounts of insoluble material were eliminated by filtration, and the filtrate was treated with magnesium oxide, according to the procedure

described by Taurog *et al.* for the separation of the choline-containing from the non-choline-containing phospholipides (7). The radioactivity,

Formation of Total Phospholipides in Liver of Rats Fed Diethanolamine (D) or Ethanolamine (E)

Series and group of rats	Diet and days on diet*	No. of rats	Sub-stance tested	Daily food intake	Change in body weight	Liver weight for 100 gm. rat	Total phospholipides	
							Radio-activity	Specific activity
Substance given in single dose								
				gm.	*gm.*	*gm.*		
I-a	26 (7)	7	None	8.9	−5	4.37 ± 0.20	394 ± 36	88 ± 4
I-b	26 (7)	6	E	9.1	−4	4.54 ± 0.16	537 ± 25	133 ± 5
I-c	26 (7)	6	D	9.3	−4	4.75 ± 0.15	689 ± 35	144 ± 13
Substance added to diet								
II-a	26 (7)	3†	None	10.3	−4	4.46 ± 0.22	439 ± 34	67 ± 6
II-b	26 (7)	2†	D‡	9.0	+3	7.22 ± 0.63	358 ± 20	36 ± 3
III-a	26 (7)	3†	None	9.0	−10	3.96 ± 0.15	472 ± 42	85 ± 6
III-b	26 (7)	4†	D‡	7.0	−12	6.46 ± 0.39	480 ± 37	43 ± 3
IV-a	26 (7)	4	None	8.9	−9	4.12 ± 0.18	594 ± 36	108 ± 6
IV-b	26 (7)	4	D	8.6	−8	5.82 ± 0.36	359 ± 24	54 ± 3
IV-c	26 (7)	4	"‡	9.5	−9	6.43 ± 0.14	567 ± 33	75 ± 2
V-a	28 (7)	4	None	7.2	−8	5.17 ± 0.17	411 ± 27	71 ± 5
V-b	28 (7)	6	E	5.3	−13	4.12 ± 0.15	450 ± 8	93 ± 3
V-c	28 (7)	4	D‡	4.8	−14	5.63 ± 0.26	484 ± 16	59 ± 5
VI-a	28 (12)	4	None	6.6	−7	5.00 ± 0.36	467 ± 47	84 ± 8
VI-b	28 (12)	6	E	5.9	−9	5.19 ± 0.28	419 ± 20	89 ± 5
VI-c	28 (12)	4	D	3.8	−17	5.19 ± 0.37	513 ± 47	71 ± 2
VI-d	28 (12)	4	"‡	3.9	−16	5.14 ± 0.18	560 ± 75	77 ± 9

The values are the averages of the data obtained on the individual livers of the rats of each group. The figures preceded by ± are the standard error of the means. The radioactivity is referred to the whole liver and expressed as per cent of the dose injected into the animal ×100. Thus, the values are directly comparable to those reported in previous papers from this laboratory, in which the radioactivity was expressed in relative radioactive units (r.r.u.), the dose injected being assumed as equal to 10^4 r.r.u. The specific activity is the radioactivity (see above) divided by the mg. of lipide P in the whole liver of a 100 gm. rat.

* The figures in parentheses indicate the days on the diet.

† Wistar rats; all other rats were of the Sherman strain.

‡ 5 minutes before the introduction of P^{32} these rats received by stomach tube an additional dose of diethanolamine (100 mg. in 1 cc. of water).

phosphorus, and often also choline (8) were determined on aliquots of the methanol solution before and after treatment with MgO, the values for the non-choline-containing fraction being obtained by difference. Prac-

tically all of the choline originally present in the lipide extracts was present in the methanol solution after treatment with MgO. However, in the latter solution the molar ratio of choline to phosphorus was often considerably below unity, which is the theoretical value in lecithins and sphingomyelins. These findings, which resemble those of our previous determinations (1), suggest either that the adsorption of non-choline-

TABLE II

Formation of Choline-Containing (CC) and Non-Choline-Containing (NCC)
Phospholipides in Liver of Rats Fed Diethanolamine (D)
or Ethanolamine (E)

Series and group of rats	Diet and days on diet*	No of rats	Substance tested	Radioactivity				Specific activity			
				Total phospholipides	CC phospholipides	NCC phospholipides	Ratio, NCC/CC	Total phospholipides	CC phospholipides	NCC phospholipides	Ratio, NCC/CC
Substance given in single dose											
I-a	26 (7)		None	350	221	129	0.59	87	89	83	0.92
I-b	26 (7)		E	530	312	218	0.70	131	133	127	0.98
I-c	26 (7)		D	615	309	306	0.98	144	122	175	1.45
Substance added to diet											
IV-a	26 (7)	4	None	541	288	253	0.88	107	101	114	1.13
IV-b	26 (7)	4	D	330	93	237	2.54	53	34	68	2.00
IV-c	26 (7)	4	"‡	454	150	304	2.02	72	50	91	1.82
VI-a	28 (12)	2	None	401	282	119	0.42	96	92	109	1.18
VI-b	28 (12)	3	E	378	215	163	0.76	81	88	72	0.82
VI-c	28 (12)	2	D	460	117	343	2.08	66	34	94	2.76
VI-d	28 (12)	2	"‡	444	132	312	2.36	78	56	94	1.68

The values for Series I are the averages of data obtained by the analysis of individual liver extracts. In the other series, duplicate determinations were carried out on pooled samples from all (Series IV) or part (Series VI) of the rats of each group. All analyses were made on the lipides redissolved in methanol, after elimination, by filtration, of the material insoluble in methanol.

See the explanations below Table I.

containing phospholipides was incomplete or that in our lipide extracts there were notable amounts of substances other than typical cephalins, which contained phosphorus but no choline.[1] Subject to these reservations, the results of the P and radioactivity determinations (Table II) show that single doses of either ethanolamine or diethanolamine stimulate

[1] The possibility should be pointed out that all or part of these substances may be formed in the analytical procedures as the result of a partial splitting of the lecithins.

the formation of both choline- and non-choline-containing phospholipides, whereas in the rats fed diethanolamine over prolonged periods the isotopic values were decreased only (radioactivities) or chiefly (specific activities) in the choline-containing fraction. In the control rats the specific activities of the two fractions were approximately equal, but in the rats which received diethanolamine, either in a single massive dose or in the diet, with or without the addition of a large dose of the amine, the specific activity of choline-containing phospholipides was much lower than that of the non-choline-containing fraction. This effect was not apparent in the rats fed ethanolamine.

Effects of Diethanolamine-Containing Diets on Lipide Composition of Liver (Table III)—In the rats on diethanolamine-containing diets the amounts of total and non-choline-containing phospholipides were considerably increased, while the values for the choline-containing phospholipides were either unchanged or lower than in the controls. Consequently, there was a marked decrease in the per cent of the choline-containing in the total phospholipides. Similar findings were not obtained when ethanolamine was added to the diet. The liver of rats fed the high fat diet with diethanolamine contained less neutral fat than that of the controls on the unsupplemented diet. However, this difference may be due to the lower intake of dietary fats rather than to a true lipotropic effect of the compound (see Handler and Dann (9)).

Attempts to Detect Incorporation of Diethanolamine in Liver Phospholipides—It was thought that some evidence on this point could be obtained by oxidizing the hydrolysate of the phospholipides with periodate and analyzing the products of this oxidation. Experiments on pure solutions showed that in the oxidation of diethanolamine for each mole of NH_3 formed 2 moles of HIO_4 were reduced and 4 moles of HCHO were formed, whereas in the oxidation of ethanolamine for each mole of NH_3 1 mole of HIO_4 was reduced and 2 of HCHO were formed.

However, one could easily anticipate that the significance of the results obtained on the hydrolysates of crude lipide extracts would be obscured by the presence in these extracts of large amounts of other products which react with periodate without yielding NH_3. A partial elimination of these interfering substances was attempted by either one of the following procedures: (a) careful purification of the phospholipides (10, 11) before hydrolysis by HCl in methanol, or (b) hydrolysis of the crude acetone precipitate, adsorption of the amines on a permutit column (12), and elution of the adsorbate with a NaCl solution. On the materials thus treated determinations of the NH_3 (13), formaldehyde (14), and periodate consumed (13) were made. From the results, it appeared that neither procedure allowed a complete elimination of the

interfering substances. However, the ratios of the NH_3 to the HIO_4 reduced and to the HCHO formed were definitely higher in the groups on diethanolamine-containing diets than in the controls on the diets alone or with added ethanolamine, the average values being 1:2.57:4.78 in the diethanolamine-fed rats (four analyses) and 1:1.52:3.54 in the con-

<div align="center">TABLE III</div>

Lipide Composition of Liver of Rats on Diets Containing Diethanolamine (D) or Ethanolamine (E)

Series and group of rats	Diet and days on diet*	No of rats	Substance added to diet	Phospholipides			Neutral fat
				Total	Choline-containing		Non-choline containing
				mg.	mg.	per cent of total	mg.	mg.
II-a	26 (7)	3†	None	149	58	39	91	121
II-b	26 (7)	2†	D‡	226	59	26	167	120
III-a	26 (7)	3†	None	126	55	44	71	111
III-b	26 (7)	4†	D‡	253	56	22	197	85
IV-a	26 (7)	4	None	125	60	48	65	299
IV-b	26 (7)	4	D	161	41	26	120	371
IV-c	26 (7)	4	"‡	176	53	30	123	305
V-a	28 (7)	4	None	139	64	46	75	539
V-b	28 (7)	6	E	112	50	45	62	299
V-c	28 (7)	4	D‡	186	50	27	136	230
VI-a	28 (12)	4	None	127	73	57	54	800
VI-b	28 (12)	6	E	103	49	48	54	694
VI-c	28 (12)	4	D	163	38	23	125	330
VI-d	28 (12)	4	"‡	165	31	19	134	267

The values have been obtained on pooled samples of the lipide extracts and are referred to the whole liver of a 100 gm. rat. Total phospholipides = mg. of P × 22.7. Choline-containing phospholipides = mg. of choline × 6.7; non-choline-containing phospholipides = total minus the choline-containing phospholipides. Neutral fat (including unsaponifiable matter) = the weight of the chloroform extract minus the weight of the total phospholipides.

See the explanations below Table I.

trols (six analyses). If these findings are accepted as presumptive evidence that diethanolamine was actually incorporated into the phospholipide molecule, one might visualize the possible formation of a peculiar type of phospholipide in which 1 molecule of diethanolamine is combined with two radicals of phosphatidic acid instead of only one. In such hypothetical compounds the ratio of N to P to fatty acids would be 1:2:4 instead of the corresponding values 1:1:2 in the natural lecithins and cephalins. To test this hypothesis, the liver phospholipides were purified

(10, 11) and their total nitrogen (micro-Kjeldahl (15)), phosphorus (6), and fatty acid (acidimetrically (16)) contents were determined. In three series of determinations, the average values for the ratios of N to P to fatty acids were 1:1.05:2.06 for the control rats and 1:1.10:2.16 for the rats on diethanolamine-containing diets. It seems, therefore, that, if diethanolamine-containing phospholipides were actually present in the liver of the rats fed diethanolamine, in these atypical phospholipides (or at least in most of them) the nitrogenous component is linked to only one radical of phosphatidic acid, as in the natural cephalins.

DISCUSSION

Previous and present results show that a single massive dose of either ethanolamine or diethanolamine causes an increase in the formation of liver phospholipides and that with either compound the increase involves both the choline-containing and the non-choline-containing phospholipides. On the other hand, when diethanolamine is added to the diet and given for longer periods, a marked decrease in the isotopic concentration of the total phospholipides is observed. This effect is chiefly the result of a decrease in the formation of choline-containing phospholipides and is peculiar for the diethanolamine-fed groups, since it was not found in the rats on a diet supplemented with ethanolamine.

The concentration of both choline-containing and non-choline-containing phospholipides in the liver is less in our control rats on low protein diets than it is in animals on stock diets (8), and, since the decrease is more marked in the former fraction, the proportion of choline-containing in total phospholipides is also decreased. Supplementation of the diets with ethanolamine did not cause an appreciable change in this picture. On the other hand, in the rats on diethanolamine-containing diets the values for the choline-containing fraction were similar to, or lower than, those in the controls, but there was a marked increase in the non-choline-containing phospholipides, so that the percentage of choline-containing in total phospholipides reached figures which are much lower than those found in rats on diets which were unsupplemented, or supplemented with the natural analogue.

Presumptive evidence was obtained for the presence of notable amounts of diethanolamine in the phospholipides of the liver of rats fed this compound. It seems, therefore, that diethanolamine can replace its natural analogue in the cephalin molecule, just as arsenocholine (17), triethylcholine (18), or sulfocholine (19) can substitute for choline as a component of the liver lecithins.

Altogether our findings may suggest that diethanolamine is easily incorporated into the phospholipides of the liver, but that these atypical

phospholipides are metabolized at a slower rate than their natural analogues; therefore the prolonged administration of diethanolamine will lead to their accumulation in the liver.

The marked inhibition of the formation of choline-containing phospholipides, observed in the rats on diethanolamine-containing diets, is a finding of considerable interest. The following hypothesis is offered as a possible explanation of the mechanism. In animals not receiving preformed choline, ethanolamine probably represents a precursor not only of cephalin but also of lecithin. The latter may be formed either indirectly, by methylation of free ethanolamine and subsequent introduction of the synthesized choline into the lecithin molecule, or directly, by methylation of the ethanolamine moiety in cephalin.[2] It seems likely that both processes would be impaired if, instead of the natural methyl acceptor, diethanolamine only were available.[3] Thus, diethanolamine would act as a metabolic antagonist of ethanolamine in the formation of both natural cephalins and lecithins.

SUMMARY

In the liver of rats maintained on low protein diets, injected with isotopic phosphate, and killed 6 hours later, the administration of a single large dose of either diethanolamine or ethanolamine stimulated lipide phosphorylation. This effect is the result of an increased formation of both choline- and non-choline-containing phospholipides. On the other hand, in the liver of rats maintained for 7 or 12 days on similar diets, supplemented with diethanolamine, the isotope content of the total phospholipides was lower than in the controls. This decrease was chiefly

[2] The assumption that cephalin is a major precursor in the formation of liver lecithin is not borne out by the finding that in the liver of normal rats (20) or dogs (21) the specific activity of the lecithin P is usually higher than that of the cephalin P. The possibility remains that the direct methylation of cephalin represents an accessory pathway for the formation of lecithin, and that this pathway might become more important in conditions in which the amount of available choline is reduced. In this respect it may be noted that in our previous (1) and present determinations on the liver of rats on choline-deficient diets we could not detect any significant or consistent difference between the specific activities of the P in the lecithin and cephalin fractions.

[3] Perhaps this effect cannot be ascribed solely to a competitive mass action between the two substances, since an actual decrease in the isotopic values of the choline-containing fraction was not observed after administration of a single large dose of diethanolamine; that is, in a condition which should lead to the greatest concentration of this substance in the tissue. In this respect, one might possibly postulate that the prolonged administration of the ethanolamine analogue results in a progressive decrease in the formation of ethanolamine from its precursors (glycine? (22)).

due to an inhibition of the formation of choline-containing phospholipides, and was not observed in experiments in which the diets were supplemented with ethanolamine.

Analysis of the lipide fractions in the liver of the rats maintained on diethanolamine-containing diets showed a considerable increase in the total and non-choline-containing phospholipides, with a marked decrease in the per cent of the choline-containing fraction. Presumptive evidence was obtained for the presence of considerable amounts of diethanolamine in the liver phospholipides of rats fed the compound. It is postulated that these atypical phospholipides are less easily metabolized than their natural analogues, and therefore accumulate in the liver. A tentative interpretation of the mechanism by which the prolonged administration of diethanolamine leads to a marked inhibition in the formation of choline-containing phospholipides is suggested.

BIBLIOGRAPHY

1. Artom, C., and Cornatzer, W. E., *J. Biol. Chem.*, **176**, 949 (1948).
2. Cornatzer, W. E., and Artom, C., *J. Biol. Chem.*, **178**, 775 (1949).
3. Fishman, W. H., and Artom, C., *J. Biol. Chem.*, **154**, 117 (1944).
4. Artom, C., and Cornatzer, W. E., *J. Biol. Chem.*, **171**, 779 (1947).
5. Artom, C., *J. Biol. Chem.*, **139**, 953 (1941).
6. Tisdall, F. F., *J. Biol. Chem.*, **50**, 329 (1922).
7. Taurog, A., Entenman, C., Fries, B. A., and Chaikoff, I. L., *J. Biol. Chem.*, **155**, 19 (1944).
8. Artom, C., and Fishman, W. H., *J. Biol. Chem.*, **148**, 405, 415, 423 (1943).
9. Handler, P., and Dann, W. J., *J. Biol. Chem.*, **146**, 357 (1942).
10. MacLean, H., *Biochem. J.*, **6**, 333, 355 (1912).
11. Le Breton, E., *Bull. Soc. chim. biol.*, **3**, 539 (1921).
12. Dubnoff, J. W., and Borsook, H., *J. Biol. Chem.*, **138**, 381 (1941).
13. Artom, C., *J. Biol. Chem.*, **157**, 585 (1945).
14. Vorländer, D., *Z. anal. Chem.*, **77**, 241 (1929).
15. Parnas, J. K., and Wagner, R., *Biochem. Z.*, **125**, 253 (1921).
16. Artom, C., *Bull. Soc. chim. biol.*, **14**, 1386 (1932).
17. Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, **35**, 107 (1936).
18. McArthur, C. S., Lucas, C. C., and Best, C. A., *Biochem. J.*, **41**, 612 (1947).
19. Maw, G. A., and du Vigneaud, V., *J. Biol. Chem.*, **176**, 1029 (1948).
20. Artom, C., Sarzana, G., Perrier, C., Santangelo, M., and Segré, E., *Arch. internat. physiol.*, **45**, 32 (1937). Chargaff, E., *J. Biol. Chem.*, **128**, 587 (1939). Haven, F. L., *J. Nat. Cancer Inst.*, **1**, 205 (1940).
21. Zilversmit, D. B., Entenman, C., and Chaikoff, I. L., *J. Biol. Chem.*, **176**, 193 (1948).
22. Stetten, D., Jr., *J. Biol. Chem.*, **140**, 143 (1941).

STUDIES ON THE ORIGIN OF THE SERUM PROTEINS*

By SIDNEY ROBERTS† AND ABRAHAM WHITE†

(From the Departments of Physiological Chemistry, Yale University, New Haven, and the School of Medicine, University of California at Los Angeles)

It has been accepted generally that the liver is the chief site of formation of certain of the plasma proteins, notably prothrombin, fibrinogen, and serum albumin. These proteins are reduced in the blood following liver damage or hepatectomy (1). On the other hand, evidence is available which suggests that reticulo-endothelial cells, other than those of the liver, may be the source of certain of the serum globulins (2, 3).

The introduction of electrophoretic methods of analysis of protein solutions has contributed an additional tool for studying the source of blood proteins. By this means, a number of investigators have determined the relative concentrations of the plasma proteins in various pathological conditions and after certain experimental procedures (*cf.* (4, 5)). The results obtained, while contributing to knowledge of variations in plasma proteins, have not provided a definite answer to the question of their origin, since a correlation has not always been possible in the intact organism between the changes observed and the activity of a particular organ or tissue.

In the present investigation, an attempt has been made to study the relative contribution by the liver and by the extrahepatic tissues to the formation of the serum proteins. The changes in the electrophoretic pattern of rat serum have been followed after partial hepatectomy and abdominal evisceration. In addition, observations have been made of protein release by surviving rat liver tissue *in vitro*. The results provide further evidence that the formation of the plasma proteins involves the integrative functioning of the liver and the extrahepatic tissues.

EXPERIMENTAL

Release of Protein by Surviving Liver Tissue in Vitro

During the course of studies on the release of antibody *in vitro* by tissue minces obtained from immunized rats and mice, it was noted that appreciable amounts of protein nitrogen were added to the serum medium (6).

Liver, spleen, kidney, and lymphoid tissue minces from non-immunized rats were also observed to release protein nitrogen when incubated in fresh rat serum under similar conditions. These surviving tissues retained their functional and histological integrity during a 3 hour incubation period in Warburg-type flasks, through which was passed a constant stream of 95 per cent oxygen-5 per cent carbon dioxide.

In the present study, liver tissue was obtained from adult, male Sprague-Dawley rats, killed by exsanguination from the abdominal aorta. Minces were prepared with the aid of a razor blade and then transferred immediately to flasks containing serum collected from the same animals supplying the tissue. In all of the experiments, 200 mg. of tissue mince were incubated in 2.0 ml. of serum at 38.5°. After incubation, the medium was centrifuged at 3000 R.P.M. for 5 minutes to remove the tissue. Total and non-protein nitrogen analyses were conducted on the serum medium before and after incubation, by a modification of the semimicro-Kjeldahl procedure described by Sobel, Yuska, and Cohen (7). Non-protein nitrogen determinations were performed after precipitation of the serum proteins with a volume of 20 per cent trichloroacetic acid equal to that of the serum sample. Total serum protein was calculated by multiplying the protein nitrogen (total nitrogen minus non-protein nitrogen) by 6.25; although this conversion factor does not necessarily give the true value for the protein content of the serum, it is useful in comparative studies.

Electrophoretic analysis of the serum medium was also conducted in each instance, prior to and following incubation. 3 ml. aliquots of the serum, pooled from two incubation flasks, were diluted with 7.5 ml. of 0.1 M sodium diethyl barbiturate buffer (pH 8.6) and dialyzed against two changes of this buffer for 2 to 3 days in the refrigerator. All electrophoretic studies were conducted at a temperature of 4°, with a constant current of approximately 15 ma. passing through the cell. The electric mobilities and percentage distribution of the protein components in serum obtained from seven control Sprague-Dawley rats, maintained on a diet of Purina laboratory chow, are shown in Table I. These data are similar to those reported by Moore et al. (8) for Long-Evans rats, and served as a base-line for most of the studies reported below.

The release of nitrogen to the serum medium during incubation of liver mince for 3 hours is depicted in Fig. 1. It is apparent that the greatest addition of protein nitrogen and of non-protein nitrogen occurred during the first half hour. Similar results have been obtained by Kline and Wilhelmi,[1] who studied the release of nitrogen by liver slices incubated in artificial media under an atmosphere of oxygen or nitrogen.

Electrophoretic examination of the serum medium before and after in-

[1] Kline, D. L., and Wilhelmi, A. E., personal communication.

cubation with liver mince for 3 hours led to the somewhat surprising observation that the protein released was in large part a component possess-

TABLE I

Electrophoretic Analysis of Serum from Adult, Male, Sprague-Dawley Rats*

Component	Electric mobility†	Percentage composition	Gm. per 100 ml. of serum‡
Albumin	6.6	50.4 ± 2.0	2.96 ± 0.25
α_1-Globulin .	5.1	19.2 ± 0.9	1.13 ± 0.11
α_2-Globulin	3.6	7.2 ± 2.2	0.42 ± 0.12
β-Globulin	2.1	14.6 ± 2.1	0.86 ± 0.11
γ-Globulin	1.8	8.6 ± 2.4	0.51 ± 0.25

* The results are expressed as the mean ± the standard deviation, wherever indicated, calculated from determinations on seven animals.

† 1×10^{-5} volt per cm.² per second.

‡ Total serum protein = 5.88 ± 0.11 gm. per 100 ml.
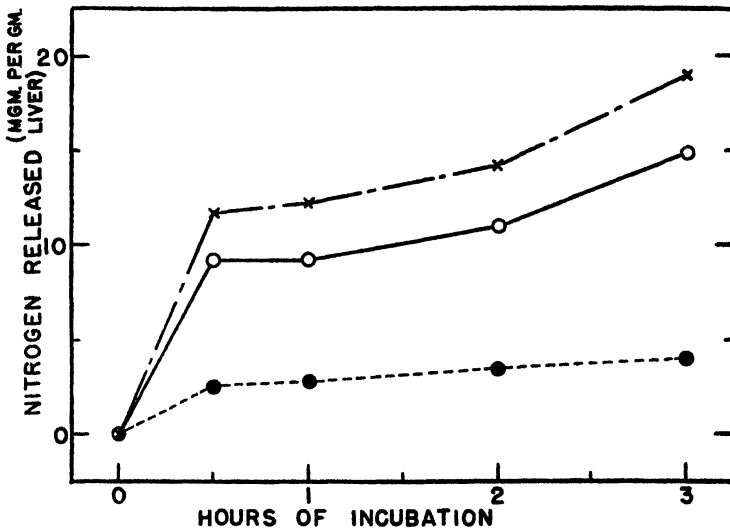
FIG. 1. The rate of release of nitrogen during incubation of surviving rat liver in rat serum. Each point represents the average amount of nitrogen released to the medium in three separate experiments. Portions of liver mince, weighing 200 mg., were incubated in 2.0 ml. of serum for periods varying from ½ to 3 hours. Nitrogen analyses of the medium before and after incubation permitted calculation of the release of total nitrogen (X), protein nitrogen (O), and non-protein nitrogen (●).

ing an electric mobility between that of serum α_1-globulin and α_2-globulin. This is illustrated in Fig. 2, in which the electrophoretic pattern of normal rat serum before incubation (A) is compared with that of the same serum

after incubation for 3 hours with rat liver mince (*B*). Calculation of the percentage distribution of the serum proteins in this typical experiment yielded the results shown in Table II. The major quantity of the added protein is included under the designation "α_1-globulin," since the limited separation of the proteins in this portion of the electrophoretic pattern did not permit satisfactory separate estimation. A marked decrease in se-

ASCENDING

(A)   (B)

DESCENDING
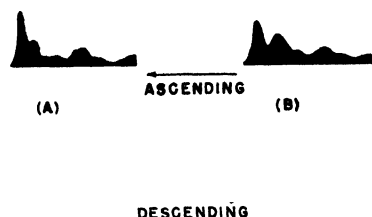
Fig. 2. Electrophoretic characterization of the protein released by liver tissue *in vitro*. Electrophoretic tracings are shown of rat serum before (*A*) and after (*B*) incubation with liver mince for 3 hours

TABLE II

Incubation of Rat Liver in Serum (Experiment 5)*

Component	Percentage composition		Gm. per 100 ml. of serum		
	Pre-incubation	Post-incubation	Pre-incubation	Post-incubation	Change
Albumin	51.8	38.7	2 90	2.49	−0.41
"α_1-Globulin"	19.2	30 8	1 08	1.98	+0.90
α_2-Globulin	7.2	7 6	0.40	0 49	+0.09
β-Globulin	15.9	16.8	0.90	1.08	+0.18
γ-Globulin	5.9	6 1	0 33	0.39	+0.06
Total			5.61	6.43	+0.82

* Incubation of 200 mg. of liver mince in 2.0 ml. of rat serum for 3 hours.

rum albumin appeared to accompany the increase in the "α_1-globulin" fraction of serum incubated with surviving liver tissue.

A composite picture of the results obtained in five incubations under an atmosphere of 95 per cent oxygen-5 per cent carbon dioxide, and in one incubation under an atmosphere of 100 per cent nitrogen, is given in Table III. It may be seen that the rate of release of liver "α_1-globulin" in oxygen varied somewhat from experiment to experiment, but was of the order of magnitude of 15 to 30 mg. per gm. of liver per hour over the 3 hour period. As has been indicated, however, about two-thirds of this protein was apparently liberated in the first half hour of incubation (Fig. 1). A decrease in serum albumin was noted in all experiments. Little or no

change occurred in globulin fractions other than "α_1-globulin." Incubation of serum alone under the same conditions produced no alteration in the serum protein pattern. Incubation of liver tissue under a nitrogen atmosphere caused changes in the serum medium which were qualitatively similar to those observed when the oxygen-carbon dioxide gas mixture was employed, but were quantitatively about 3-fold greater (Table III). The increased release of tissue nitrogen during incubation in a nitrogen atmosphere has been observed earlier by Bernheim and Bernheim (9) for surviving liver and kidney slices. In the present instance, it is interesting to note that in the nitrogen atmosphere there was also an exaggerated loss of albumin, suggesting that the processes involved in

TABLE III

Release of Protein to Serum Medium during Incubation of Surviving Rat Liver*

Component	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6†	Average, Experiments 1-5
Albumin.	−2.3	−4.7	−2.0	−17.3	−13.7	−29.8	−8.0
"α_1-Globulin"	16.3	18.1	20.7	25.0	30.0	59.3	22.2
α_2-Globulin . .	2.1	−0.8	−2.0	4.3	2.0	1.6	1.1
β-Globulin .	0	3.0	−0.3	−0.3	6.0	7.2	1.7
γ-Globulin....	−0.8	1.1	−0.7	0.3	2.0	−1.6	0.4
Total protein.. .	15.3	16.7	15.7	12.0	27.3	36.7	17.4

* Expressed as mg. of protein added to the serum per gm. of liver per hour and representing the average value during a 3 hour incubation period.

† All incubations were conducted under an atmosphere of 95 per cent O_2-5 per cent CO_2, except Experiment 6, in which an atmosphere of 100 per cent N_2 was employed.

the loss of albumin and gain of "α_1-globulin" occurred under anaerobic, as well as aerobic, conditions and might be causally related.

Changes in Serum Proteins after Partial Hepatectomy

Partial hepatectomy was performed (10) on adult, male Sprague-Dawley rats. Immediately after operation, a blood sample was taken from the tail of each rat for analysis of serum total and non-protein nitrogen; these data served as the control values for assessing changes in total serum protein. The animals were then placed in individual cages and permitted to feed ad libitum on Purina laboratory chow, with free access to drinking water. At periods of time varying from 0 to 48 hours after operation, the rats were sacrificed in pairs by exsanguination from the abdominal aorta. Pooled sera from two animals were used for nitrogen determinations and electrophoretic analysis.

In Fig. 3 the electrophoretic pattern of the serum pooled from two rats immediately after partial hepatectomy (A) is compared with the patterns observed in similar samples taken from separate pairs of partially hepatectomized animals at intervals of 6 hours (B), 12 hours (C), 16 hours (D), 24 hours (E), and 48 hours (F) after operation. It will be noted that all of the serum proteins exhibited decreases within the 48 hour experimental period after partial hepatectomy. Fig. 4 reveals that the earliest and most extensive postoperative alteration in serum proteins was a decrease in the α_1-globulin fraction, which at 12 hours after partial hepatectomy had declined to approximately one-half its normal value. By 16 hours, at a time corresponding to the onset of active liver regeneration (11), the α_1-globulin fraction had begun to return to the preoperative value. The other serum globulins declined slowly for 24 hours, and then began to return to the normal level, which was reached, in most in-
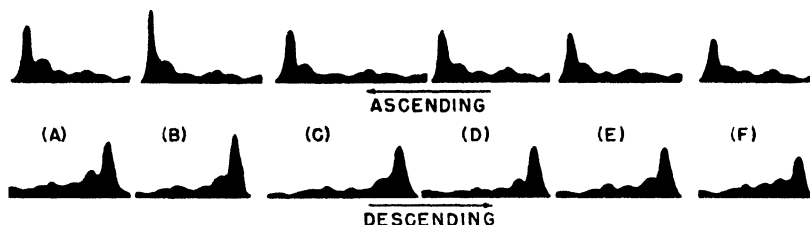
Fig. 3. Electrophoretic changes in rat serum after partial hepatectomy. Tracings are shown of the electrophoretic patterns of pooled serum obtained from two rats at each of the following intervals (in hours) after partial hepatectomy: 0 (A), 6 (B), 12 (C), 16 (D), 24 (E), and 48 (F).

stances, by the end of the 48 hour experimental period. The alterations in serum albumin were correlated inversely with those in α_1-globulin; albumin values showed an early rise, followed by a later and prolonged fall.

The decline in total serum protein following partial removal of the liver was maximal about 24 hours after operation, and amounted to 1.20 gm. per 100 ml. This represents a decrease of approximately 20 per cent below normal values (cf. Table I). It may be noted that control rats fasted for 1 to 2 days showed no significant changes in serum proteins. This is of importance inasmuch as the rat ingests very little food during the initial 12 to 18 hour period following partial hepatectomy (cf. (11)).

The serial alterations in serum proteins described above apparently were not due to hemodilution or hemoconcentration. Hemoglobin determinations conducted on aliquots of whole blood samples over the 2 day period following partial removal of the liver revealed no significant changes. Moreover, alterations in blood volume or blood concentration could not explain the serum protein data which were obtained, since the trends in
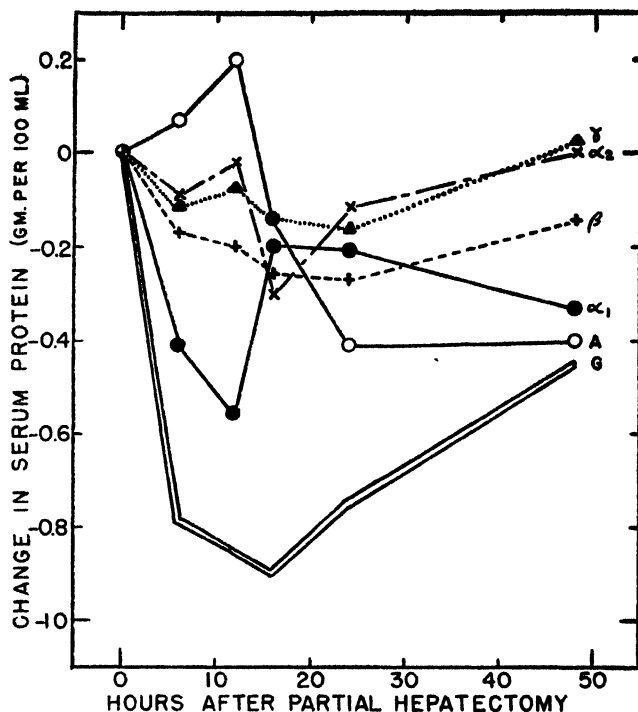
Fig. 4. The change in serum proteins after partial hepatectomy. Each point (except the zero point) represents the results of electrophoretic analysis of two pools of sera obtained from at least four animals partially hepatectomized 6 to 48 hours earlier. Total serum proteins were determined on each animal at the time of operation and of autopsy. The zero point for each rat was calculated from the electrophoretic results depicted in Table I, combined with the actual serum protein level measured in the particular animal before operation. Albumin is represented by A, total globulin by G, and the various individual globulins by the designations on the curves

ASCENDING

(A)   (B)   (C)   (D)

DESCENDING
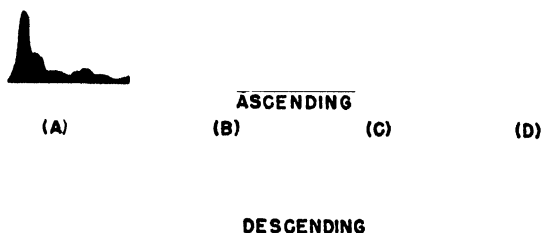
Fig. 5. Electrophoretic changes in rat serum after abdominal evisceration. Tracings are shown of pooled serum obtained from two rats at each of the following intervals (in hours) after evisceration: 0 (A), 10 (B), 18 (C), 23 (D).

values for albumin and the globulins were in opposite directions over a large portion of the experimental period.

Changes in Serum Proteins after Abdominal Evisceration

In order to assess the possible influence of liver regeneration on the serum protein alterations observed in the previous experiments, experi-
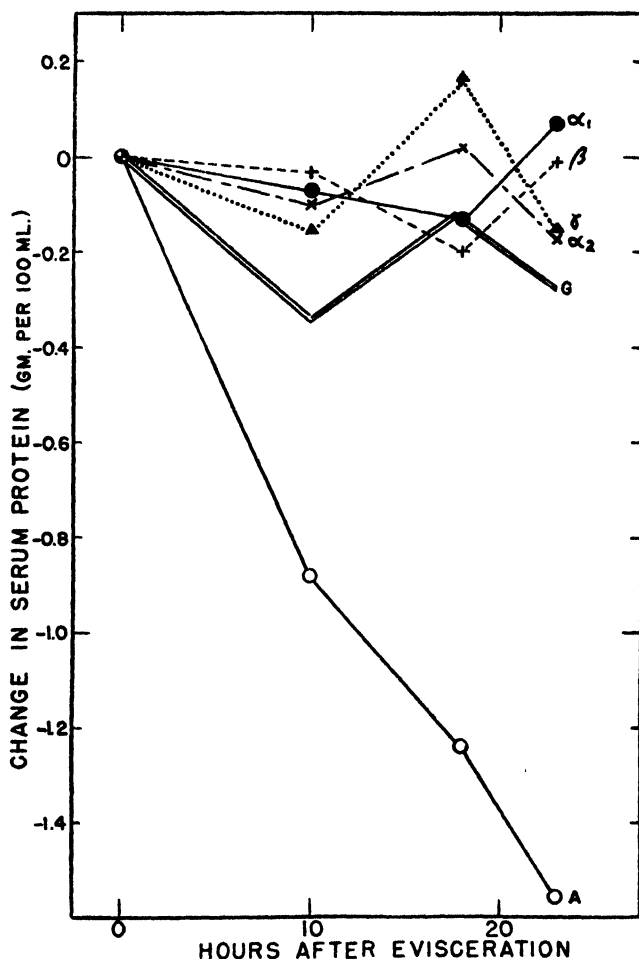
FIG. 6. The change in serum proteins after abdominal evisceration. See the legend to Fig. 4 for experimental details and other explanations. The animals were sacrificed at intervals varying from 10 to 23 hours after operation.

ments were performed in which the liver of the rat was removed completely. In this operation (12), the gut and its associated structures were also removed, so that any effects noted must be related to the absence of these tissues, as well as of the liver. All animals were fasted 24 to 48

hours prior to evisceration, since this lengthens their postoperative life span (13). As noted above, this period of fasting had no demonstrable effect on serum protein levels.

Figs. 5 and 6 illustrate the results obtained by analyses at varying intervals after evisceration. Fig. 5 depicts the electrophoretic pattern observed in serum pooled from two rats immediately after operation (A), and 10 hours (B), 18 hours (C), and 24 hours (D) later. The changes in the concentrations of the various serum proteins are shown in Fig. 6.

The maximal decline in total serum proteins was observed 23 hours after evisceration and amounted to approximately 35 per cent of normal. Beyond this time, those animals which survived were too moribund to provide sufficient blood for electrophoretic characterization. It is noteworthy that the decrease in serum proteins in the eviscerated rat was due almost entirely to an immediate and profound fall in serum albumin. The results presented in Fig. 6 are corrected for the hemoconcentration which occurred; this reached a value of 15.7 per cent above normal at the end of the 23 hour experimental period. Protein nitrogen could not be detected in the bladder urine, indicating no significant loss of albumin through the kidney.

DISCUSSION

The extensive work of Whipple and his associates has implicated the liver as the principal source of the blood proteins (cf. (1, 14)). These investigators have suggested that a major portion of the protein synthesized in the body is formed in this organ (presumably from amino acids), liberated to the blood as plasma proteins, and utilized by other tissues for the formation of their specific cell proteins. However, Tarver and Reinhardt (15) demonstrated that, in the hepatectomized dog, radioactive sulfur (administered intravenously as methionine) was incorporated at a normal rate into the proteins of all tissues examined except the blood.

The observation by Munro and Avery (16) that all of the plasma proteins declined after hepatectomy in the dog would seem to point to the liver as the source of the blood globulins as well as albumin. Several lines of investigation, however, suggest that hepatic tissue is concerned more specifically with the formation of plasma albumin than of globulin. Thus, in severe protein depletion (1, 17, 18), and after partial removal of the liver in the rat ((19) and present study), plasma albumin values have been shown to undergo a marked and prolonged reduction. Under similar circumstances, certain of the blood globulins may even be elevated (17, 19, 20). In addition, the reticuloendothelium has long been implicated in the formation of antibody globulin; this evidence has been reviewed recently by Wilson and Miles (21). The increased plasma globulin con-

centrations seen frequently in multiple myeloma (*cf.* (4, 5)) also suggest that blood protein may be derived from extrahepatic tissues. Recent work has indicated that the lymphoid structures may be an important source of normal and immune γ-globulin (3, 22–24), and perhaps, of β-globulin as well (3, 25).

In the data of Tarver and Reinhardt (15), the rate of incorporation of labeled sulfur into blood globulin in the hepatectomized dog was one-seventh that of control animals; for albumin, this figure was only one-twentieth the control value. In the present investigation, the profound drop in serum albumin after abdominal evisceration, notwithstanding minimal changes in the globulins, would also appear to favor the hypothesis that the formation of serum albumin is more dependent on the presence of an intact liver than is the synthesis of serum globulin. The constancy of the serum globulin levels in the eviscerated rat supports the concept of an extrahepatic origin of a significant portion of the blood globulins. However, an alternative explanation for these data could be based on a preferential utilization of albumin by the peripheral tissues, accompanied by a cessation of removal of globulin from the blood.

The liberation of "α_1-globulin" from liver mince *in vitro*, correlated with a disappearance of albumin from the serum medium, may represent a phenomenon of physiological importance. Thus, it may be noted that the levels of α_1-globulin and albumin exhibited an inverse correlation in the partially hepatectomized rat, one falling while the other rose, and vice versa. Moreover, a number of investigators (*cf.* (26–28)) have directed attention to the fact that conditions which result in a depression in serum albumin (*e.g.*, protein depletion, tissue injury, infection, etc.) are frequently accompanied by a rise in the α-globulin peak in the electrophoretic patterns of plasma in all species of animals examined. In certain instances (29, 30), part of this apparent increase in protein may be due to a lipemic response to the stress, in which a significant portion of the increased plasma lipide forms a lipoprotein complex with α-globulin. It would appear likely, then, that α-globulin may represent a protein readily released to the blood by the liver in response to many different types of stress or tissue injury. This conclusion is substantiated by the fact that increases in α-globulin in the blood do not accompany the depression in albumin observed in animals completely deprived of their livers; *i.e.*, hepatectomy in the dog (16) and evisceration in the rat (present study). Moreover, the correlation between the onset in liver regeneration and the return of α-globulin to normal levels in the partially hepatectomized rat is further evidence for the hepatic origin of this protein.

The present study suggests either that albumin may have been the source of part of the "α_1-globulin" liberated by the liver, or provided en-

ergy for "α_1-globulin" formation. A further explanation for the decrease in albumin is provided by the observation, made in the course of these studies, that large amounts of glucose were added to the incubation medium in the *in vitro* experiments. This occurred even though the liver was obtained from animals fasted 24 hours and was therefore depleted of glycogen. In one experiment, the liver mince added 12.25 mg. of glucose per gm. of liver per hour to the incubation medium, indicating that this glucose was derived from non-carbohydrate sources (*cf.* also Shipley (31)). Since in these experiments there was an actual increase in the amino acid content of the serum, the data may reflect the capacity of the liver to effect gluconeogenesis from serum albumin *in vitro*.

SUMMARY

Incubation of surviving liver mince in rat serum, under an atmosphere of 95 per cent oxygen-5 per cent carbon dioxide, resulted in the liberation of large amounts of a protein migrating with an electric mobility approximating that of α_1-globulin. The amount of this protein liberated during a 3 hour incubation period varied from 15 to 30 mg. per gm. of tissue per hour. The major portion of the protein was apparently released during the first half hour of incubation. An increased rate of liberation of "α_1-globulin" occurred when a nitrogen atmosphere was employed during the incubation period. In all experiments, the release of "α_1-globulin" was accompanied by a disappearance of albumin from the serum medium.

Alterations in serum proteins have been examined in the rat after partial hepatectomy and abdominal evisceration. The value for total serum protein was maximally depressed approximately 24 hours after partial hepatectomy; it decreased 1.2 gm. per 100 ml. of serum. Electrophoretic examination revealed that the earliest protein decrease occurred in the α_1-globulin fraction, followed by later declines in albumin and in the other serum globulins. Approximately 16 hours after partial hepatectomy, the globulins, and especially α_1-globulin, began a rapid return to normal levels. Albumin levels, however, remained low over the 48 hour experimental period. Following abdominal evisceration in the rat, the serum protein level decreased rapidly over the entire 23 hour period of observation. The decline for this period was of the order of 2.0 gm. per 100 ml. of serum. Electrophoretic analysis showed the total protein decrease to be due almost entirely to a lowering of the serum albumin value.

The data suggest that the formation of serum albumin and α_1-globulin occurs largely in the liver, whereas the synthesis of other serum globulins can be accomplished by extrahepatic tissues.

BIBLIOGRAPHY

1. Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, **20**, 194 (1940).
2. Sabin, F. R., *J. Exp. Med.*, **70**, 67 (1939).
3. White, A., and Dougherty, T. F., *Ann. New York Acad. Sc.*, **46**, 859 (1946).
4. Luetscher, J. A., Jr., *Physiol. Rev.*, **27**, 621 (1947).
5. Gutman, A. B., *Advances in Protein Chem.*, **4**, 156 (1948).
6. Roberts, S., Adams, E., and White, A., *J. Immunol.*, **62**, 155 (1949).
7. Sobel, A. E., Yuska, H., and Cohen, J., *J. Biol. Chem.*, **118**, 443 (1937).
8. Moore, D. H., Levin, L., and Leathem, J. H., *J. Biol. Chem.*, **153**, 349 (1944).
9. Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **163**, 203 (1946).
10. Roberts, S., and Szego, C. M., *Endocrinology*, **40**, 73 (1947).
11. Szego, C. M., and Roberts, S., *J. Biol. Chem.*, **178**, 827 (1949).
12. Reinecke, R. M., *Am. J. Physiol.*, **140**, 276 (1943).
13. Reinecke, R. M., and Roberts, S., *Am. J. Physiol.*, **141**, 476 (1944).
14. Whipple, G. H., Hemoglobin, plasma protein, and cell protein, Springfield (1948).
15. Tarver, H., and Reinhardt, W. O., *J. Biol. Chem.*, **167**, 395 (1947).
16. Munro, M. P., and Avery, A., *Am. J. Physiol.*, **146**, 673 (1946).
17. Chow, B. F., Allison, J. B., Cole, W. H., and Seeley, R. D., *Proc. Soc. Exp. Biol. and Med.*, **60**, 14 (1945).
18. Elman, R., Heifetz, C. J., and Wolf, H., *J. Exp. Med.*, **73**, 417 (1941).
19. Chanutin, A., Hortenstine, J. C., Cole, W. S., and Ludewig, S., *J. Biol. Chem.*, **123**, 247 (1938).
20. Berryman, G. H., Bollman, J. L., and Mann, F. C., *Am. J. Physiol.*, **139**, 556 (1943).
21. Wilson, G. S., and Miles, A. A., Topley and Wilson's Principles of bacteriology and immunity, Baltimore, 3rd edition (1946).
22. Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E., *J. Exp. Med.*, **81**, 73 (1945).
23. Kass, E. H., *Science*, **101**, 337 (1945).
24. Mondolfo, H., and Hounie, E., *Dia med.*, **19**, 1112 (1947).
25. Roberts, S., and White, A., *J. Biol. Chem.*, **178**, 151 (1949).
26. Zeldis, L. J., Alling, E. L., McCoord, A. B., and Kulka, J. P., *J. Exp. Med.*, **82**, 157 (1945).
27. Chow, B. F., *Ann. New York Acad. Sc.*, **47**, 297 (1946).
28. Gjessing, E. C., Ludewig, S., and Chanutin, A., *J. Biol. Chem.*, **174**, 683 (1948).
29. Zeldis, L. J., Alling, E. L., McCoord, A. B., and Kulka, J. P., *J. Exp. Med.*, **82**, 411 (1945).
30. Chanutin, A., and Gjessing, E. C., *J. Biol. Chem.*, **178**, 1 (1949).
31. Shipley, R. A., *Am. J. Physiol.*, **141**, 662 (1944).

THE SUBSTRATE SPECIFICITY AND SEDIMENTATION
BEHAVIOR OF δ-CHYMOTRYPSIN

BY GEORGE W. SCHWERT AND SEYMOUR KAUFMAN*

(From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina)

In 1947 Jacobsen (1) reported that, when chymotrypsinogen is activated at 0° by a much larger amount of trypsin than was specified by Kunitz and Northrop (2) for the preparation of α-chymotrypsin, the resultant solution has a specific activity about 50 per cent greater than that of crystalline α-chymotrypsin. From a kinetic analysis of the activation process, Jacobsen concluded that tryptic hydrolysis of one peptide bond per chymotrypsinogen molecule yields π-chymotrypsin, a hitherto unknown protease, having a specific activity 2 to 2.5 times that of α-chymotrypsin. It was further concluded that π-chymotrypsin is destroyed by two competitive reactions: (a) the tryptic hydrolysis of one peptide bond per molecule to yield a second new enzyme, δ-chymotrypsin, with an activity about 1.5 times that of α-chymotrypsin, and (b) autolytic or spontaneous hydrolysis of three peptide bonds per π-chymotrypsin molecule to yield α-chymotrypsin. π-Chymotrypsin is so unstable that the maximum measurable activity obtained by the rapid activation of chymotrypsinogen is the specific activity of δ-chymotrypsin.

As a logical consequence to studies of the kinetics of the hydrolysis of specific esters and amides by α-chymotrypsin (3) and of the sedimentation rates of chymotrypsinogen, α-chymotrypsin, and γ-chymotrypsin (4), the present paper extends these investigations to δ-chymotrypsin.

EXPERIMENTAL

δ-Chymotrypsin—1.1 gm. of lyophilized, essentially salt-free, chymotrypsinogen[1] (prepared from chymotrypsinogen recrystallized eight times) were dissolved in 50 ml. of water. A small trace of insoluble material was filtered off and the pH of the solution was adjusted to 7.3 with 0.1 N NaOH. The solution was placed in a refrigerated bath at 0°. After 20 minutes 70 mg. of crystalline trypsin (containing approximately 50 per cent $MgSO_4$) were added. According to Jacobsen (1) maximum activity should be attained in 1 to 2 hours under these conditions.

Samples were withdrawn after 30, 67, and 91 minutes and the activity

---

against a constant concentration of acetyl-L-tyrosine ethyl ester was determined. Constant values corresponding to about twice the activity of α-chymotrypsin were obtained with all of these samples.

After a total of 98 minutes the pH of the activation solution was rapidly adjusted to pH 4.2 with 2 N H_2SO_4 and the solution was shell-frozen and lyophilized. The dry protein was stored at $-20°$. Over a period of about 2 months no change in the activity of this preparation was observed.

Substrates—The preparation of the substrates used in this study has been described elsewhere (3, 5).

Methods—The methods used for determining amidase and esterase activity have been described (6) as have the procedures used in the ultra-centrifugal study (4).

Enzyme concentrations for the enzymatic studies were determined by the semimicro-Kjeldahl method. Apparently the chymotrypsinogen from which the δ-chymotrypsin was prepared still contained a small amount of ammonium salts, since the blanks in the Conway method for the amide substrate were uniformly high and varied with enzyme concentration. These blanks, corrected for the ammonia content of the other reagents, were used to correct the Kjeldahl analyses. Correction was made for the small amount of nitrogen contributed by the trypsin used in the activation.

The protein concentrations for the sedimentation analyses were determined by the method of Kunitz (7) with the Beckman spectrophotometer. No attempt was made to establish a calibration curve for the rather poorly characterized δ-chymotrypsin. Protein concentrations were read from the calibration curve for α-chymotrypsin. This procedure should result in little or no error, since the amino acid content of the two chymotrypsins is probably identical (1, 8).

Results

Kinetic Studies

Acetyl-DL-tryptophan Ethyl Ester (ATrEE) and Acetyl-L-tyrosine Ethyl Ester (ATEE)—The hydrolysis of these substrates was studied in 30 per cent methanol as a function of substrate concentration. Initial substrate concentrations were determined as previously described (3). The reactions follow apparent first order kinetics. For ATrEE with δ-chymotrypsin, as with α-chymotrypsin, hydrolysis proceeds to 50 per cent of completion with respect to the concentration of the racemate. In accordance with previous studies on the antipodal specificity of chymotrypsin, it may be assumed that only the L form of the substrate was hydrolyzed.

K_m was determined from the plot of the Lineweaver-Burk equation (9), $a/v = K_m/V_{max.} + a/V_{max.}$, where a is the substrate concentration and v

TABLE I

Comparative Kinetic Constants for Hydrolysis of ATA, ATrEE, and ATEE by α-Chymotrypsin and δ-Chymotrypsin[*]

Enzyme	Substrate	K_m	k'[†]	C_{max}[‡]
		$\mu \times 10^3$	$\times 10^3$	$\times 10^3$
α-Chymotrypsin	ATA	27	3.0	4.8
	ATrEE	1.7	320	8,300
	ATEE	32	2600	3,600
δ-Chymotrypsin	ATA	2.0	4.5	96
	ATrEE	0.36	400	49,000
	ATEE	16	2900	7,700

[*] The kinetic constants for the hydrolysis of these substrates by chymotrypsin differ slightly from those previously reported (3, 5). The value of v used in the Lineweaver-Burk equation (9) must be determined at a time interval small enough so that deviations from linearity in the course of the reaction are negligible compared to the error of the experimental measurements. The values previously reported were calculated with v_5 min for ATEE and v_1 min for ATrEE and ATA. Initial reaction velocities for the data shown in Table I were obtained by plotting the experimental data according to any function which gave a straight line over most of the reaction course (see the text), and from this function the slope of the reaction curve at zero time was calculated. These values are believed to represent a better approximation of the kinetic constants than do those previously reported.

[†] k' is the rate of the slowest step between the formation of the enzyme-substrate complex and the subsequent appearance of free enzyme and reaction products. It has the dimensions, moles hydrolyzed per liter per minute per mg. of enzyme N per ml.

[‡] C_{max} = $k'/2.3K_m$ and is the maximum proteolytic coefficient, assuming adherence of the reaction to first order kinetics (10).

TABLE II

Sedimentation Analyses of δ-Chymotrypsin

The symbols used are those defined by Svedberg and Pedersen (11).

pH[*]	Concentration of protein	s	Temperature	s'_{20}	$s_{20, w}$[†]
	mg. per ml.	Svedberg units	°C	Svedberg units	Svedberg units
3.86	0.94	2.76	25.2	2.44	2.54
3.86	4.10	2.89	25.2	2.55	2.68
3.86	8.31	2.76	24.2	2.49	2.60
4.99	2.03	2.84	25.3	2.50	2.62
4.99	15.4	3.01	25.7	2.64	2.74
6.20	3.90	2.96	25.9	2.58	2.66
6.20	11.4	3.21	25.9	2.78	2.88

[*] All buffers had an ionic strength of 0.20 and contained 0.18 M NaCl. Acetate buffers were used for pH 3.86 and 4.99 and phosphate for pH 6.20.

[†] The partial specific volume was taken as 0.73, the value found for chymotrypsinogen.

is the rate of decomposition. k' was determined as described previously (10).[3] The results of these calculations are shown in Table I.

Acetyl-L-*tyrosinamide* (*ATA*)—The hydrolysis of this substrate was studied in aqueous solution at varying initial substrate concentrations. Kinetic constants were calculated by the methods used for ATrEE.

Although the hydrolysis of ATA by α-chymotrypsin follows apparent first order reaction kinetics, it was found that with δ-chymotrypsin the rate decreased more rapidly than is required by first order kinetics. The expected increase in initial slope of the first order plot with decreasing substrate concentration was observed, but the deviation from linearity of this plot also increased with decreasing substrate concentration.

When $1/x$ was plotted against $1/t$ when x = moles of substrate per liter hydrolyzed in time t, straight lines were obtained, and from these lines calculation of the value of dx/dt at zero time was possible.

The results of these calculations together with comparable data for α-chymotrypsin are shown in Table I.

The stability of δ-chymotrypsin in acid solution was determined by allowing a solution containing 3 mg. of protein in 100 ml. of 0.004 N HCl to stand at refrigerator temperature. Four samples withdrawn 11, 168, 328, and 1368 minutes after the solution was made up showed identical activity against acetyl-L-tyrosine ester.

Determination of Sedimentation Constant

The results of the sedimentation analyses on δ-chymotrypsin, made in the electrically driven ultracentrifuge (Specialized Instruments Corporation), are shown in Table II. At the three pH values used for these determinations, all boundaries were highly symmetrical. In no case was any trace of a second component observed.

DISCUSSION

In a comparative study of this kind, in which enzymes having the same origin and specificity but different activities are studied, four results seem possible.

(*a*) δ-Chymotrypsin has more active centers per molecule than does α-chymotrypsin, but the properties of each active center are identical in both enzymes. In this case K_m should be essentially unchanged and k' should be greater for δ- than for α-chymotrypsin. This will be true, since K_m is independent of enzyme concentration, while k' is a function of enzyme concentration.

(*b*) Similar kinetic results would be found if the number of active

[3] Defined in note to Table I.

centers was the same for both enzymes, but the enzyme-substrate activation process proceeded at a higher rate for δ- than for α-chymotrypsin. In this case, however, since $K_m = (k_2 + k')/k_1$, where k_1 is the velocity constant for the formation of the enzyme-substrate complex from free enzyme and substrate, k_2 is the constant for the dissociation of this complex, and k' is the rate of activation of the enzyme-substrate complex, K_m would be expected to exhibit some increase due to the increase in k'.

(c) The rate of activation is identical for both enzymes but the affinity of δ-chymotrypsin for the substrates is much higher than that of α-chymotrypsin. In this case K_m should decrease and k' should be constant.

(d) The enzymatically active centers are so modified in δ-chymotrypsin that both enzyme-substrate affinity and activation rate are increased over the corresponding values for α-chymotrypsin.

The data shown in Table I seem to support case (d). Both the enzyme-substrate affinity and the rate of activation of the enzyme-substrate complex are greater for δ- than for α-chymotrypsin. However, it appears that the increased over-all reaction rate observed with δ-chymotrypsin depends much more upon the large change in K_m than on the relatively small change in k'. In this connection it is of interest that the smaller the value of k' for a substrate hydrolyzed by α-chymotrypsin, the greater is the increase in k' when this substrate is hydrolyzed by δ-chymotrypsin. With ATEE, the most reactive known substrate of chymotrypsin, k' is essentially constant for both enzymes. This suggests that, when this rate of activation has been attained, there is some other limiting process than the activation *per se*. This limiting step could be the rate of entry of water into the reaction or the rate of desorption of the reaction products from the enzyme surface.

Although it has been shown for α-chymotrypsin that k' is independent of methanol concentration and that K_m increases with methanol concentration (12), it is possible that the effect of methanol on K_m differs for the two chymotrypsins.[3] In any case it appears that the active centers on the δ-chymotrypsin molecule differ from those on the α-chymotrypsin molecule. If this formulation of the hydrolytic process is correct, the concept of homospecificity of enzymes, proposed by Irving, Fruton, and Bergmann (13) must be regarded only as a first approximation. This view-point has been previously expressed (3, 10).

Other conclusions which can be drawn from the kinetic data are as follows:

1. The two enzymes presumably have the same optical specificity,

[3] If the effect of methanol on the chymotryptic hydrolysis of ATA is the same for both enzymes (12), $k'_\delta/k'_\alpha = 1.5$, whereas $k_{1\delta}/k_{1\alpha} = 20$ (cf. Table I).

since the hydrolysis of acetyl-DL-tryptophan ethyl ester proceeds to 50 per cent of completion with each enzyme, while each hydrolyzes acetyl-L-tyrosinamide and acetyl-L-tyrosine ethyl ester completely.

2. The structural specificity of the two enzymes is qualitatively the same toward amide and ester substrates.

3. The observed deviations of the δ-chymotrypsin-ATA system from first order reaction kinetics suggest that δ-chymotrypsin is highly unstable in the pH range in which it is enzymatically active at 25°. The protective action of the substrate seems to account for the smaller deviations observed at higher substrate concentrations. This lack of stability was not observed in the determination of esterase activity, probably because the esterase activity determinations were carried out over a period of 10 to 15 minutes, while the amidase activity determinations were carried out over a 70 minute period. Inasmuch as the activity of the enzyme preparation was found to remain constant for an hour at 0° and pH 7.3 during the activation process, it appears that the temperature coefficient for the inactivation of δ-chymotrypsin must be large.

The sedimentation studies in the ultracentrifuge indicate that δ-chymotrypsin is intermediate in its behavior between chymotrypsinogen and α-chymotrypsin. Chymotrypsinogen shows, within the limits of error of the measurements, "normal" behavior, i.e., $s_{20, w}$ decreases slightly with increasing protein concentration. α-Chymotrypsin, on the other hand, exhibits a marked increase in $s_{20, w}$ as protein concentration is increased in the concentration range below 1 per cent. δ-Chymotrypsin shows a small increase in $s_{20, w}$ with increasing protein concentration, but, from the four points representing the lowest protein concentrations, the extrapolation to zero concentration seems reasonably clear. $s_{20, w}$, extrapolated to zero protein concentration, is 2.5 (2.53) Svedberg units. The value for chymotrypsinogen was found to be 2.7 Svedberg units, while that for chymotrypsin seems to be about 2.5 Svedberg units (4).

SUMMARY

The kinetics of the hydrolysis of acetyl-L-tyrosinamide, acetyl-L-tyrosine ethyl ester, and acetyl-DL-tryptophan ethyl ester by δ-chymotrypsin was studied and compared to the kinetics of the hydrolysis of these substrates by α-chymotrypsin. Both enzyme-substrate affinity and activation rate are greater with δ-chymotrypsin than with α-chymotrypsin.

The sedimentation constant of δ-chymotrypsin, extrapolated to zero protein concentration, was found to be 2.5 Svedberg units.

BIBLIOGRAPHY

1. Jacobsen, C. F., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **25**, 325 (1947).
2. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **19**, 991 (1936).
3. Kaufman, S., Neurath, H., and Schwert, G. W., *J. Biol. Chem.*, **177**, 793 (1949).
4. Schwert, G. W., *J. Biol. Chem.*, **179**, 655 (1949).
5. Kaufman, S., and Neurath, H., *Arch. Biochem.*, **21**, 437 (1949).
6. Schwert, G. W., Neurath, H., Kaufman, S., and Snoke, J. E., *J. Biol. Chem.*, **172**, 221 (1948).
7. Kunitz, M., *J. Gen. Physiol.*, **30**, 291 (1947).
8. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **18**, 433 (1934).
9. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
10. Elkins-Kaufman, E., and Neurath, H., *J. Biol. Chem.*, **175**, 893 (1948).
11. Svedberg, T., and Pedersen, K. O., The ultracentrifuge, Oxford (1940).
12. Kaufman, S., and Neurath, H., *J. Biol. Chem.*, **180**, 181 (1949).
13. Irving, G. W., Jr., Fruton. J. S., and Bergmann, M., *J. Biol. Chem.*, **138**, 231 (1941).

OXYBIOTIN DERIVATIVES AS BIOTIN AND OXYBIOTIN ANTAGONISTS*

By A. E. AXELROD and KLAUS HOFMANN

(From the Department of Chemistry, University of Pittsburgh, and the Institute of Pathology, Western Pennsylvania Hospital, Pittsburgh)

Biotin antagonists have been investigated in this laboratory both from the standpoint of their potential chemotherapeutic value and their application to studies of biotin metabolism. The synthesis of a number of oxybiotin derivatives (1–3) and their use in studies relating to the enzymatic function of biotin (4) have been reported. The present paper describes in detail the effects of these compounds upon the growth of several microorganisms.

Oxybiotin, rather than biotin, derivatives were used, since oxybiotin was found to possess biotin-like activity (5, 6) and the preparation of such compounds had already been well developed by us.

Preparation of Compounds

Compounds I to XVI (Table I) were prepared as described in previous communications (1–3). cis-3,4-Diamino-2-tetrahydrofurancaproic acid sulfate (Compound XVII) was prepared from dl-homooxybiotin (Compound IV) by barium hydroxide hydrolysis (7). The salt was purified by crystallization from aqueous methanol and melted at 225–227°.

$C_{10}H_{22}O_7N_2S$. Calculated. C 38.21, H 7.05, N 8.91, S 12.84
Found. " 38.27, " 7.31, " 8.77, " 12.54

Saccharomyces cerevisiae, 139,[1] *Lactobacillus arabinosus*, 17-5,[1] *Streptococcus hemolyticus*, Lancefield group C, mucoid, Stoddard strain (8), *Escherichia coli*, 9723,[1] and *Staphylococcus aureus*, 6538,[1] were employed as the test organisms.

Culture Methods

S. cerevisiae—The stock culture was kept at 30° on Difco wort agar slants. To prepare an inoculum, the organisms were removed from a 24 hour slant and suspended in normal saline. 0.1 cc. of this suspension (containing 0.01 mg. of dry yeast) was used to inoculate 10 cc. of the Snell,

[1] Obtained from the American Type Culture Collection.

TABLE I

Antibiotin and Antioxybiotin Activities of Oxybiotin Derivatives
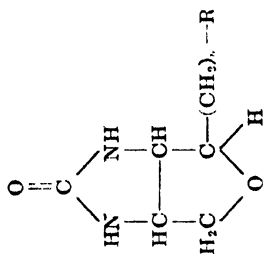
The values given are those for the antibacterial indices. The percentage activities compared to *dl*-oxybiotin are given in parentheses for those compounds which were growth-promoting. All the compounds were of the *dl* form.

Compound No.		n	R	S. cerevisiae		L. arabinosus		S. hemolyticus	
				d-Biotin	*dl*-Oxybiotin	*d*-Biotin	*dl*-Oxybiotin	*d*-Biotin	*dl*-Oxybiotin
I	*bis*-Noroxybiotin	2	—COOH	>500,000	135,000		(0.00030)		(0.0028)
II	Noroxybiotin	3	"	>500,000	143,000		(0.00044)	>2,000,000	125,000
III	Oxybiotin	4	"	(100)		(100)		(100)	
IV	Homooxybiotin	5	"	445,000	7,000	>500,000	225,000	1,250,000	5,800
V	*bis*-Homooxybiotin	6	"	>500,000	30,000		(0.0033)	1,000,000	25,000
VI	Hexahydro-2-oxo-4-(4,4-dicarboxylbutyl)-1-furo-3.4-imidazole	3	COOH —CH—CH	(2.9)		(1.0)			
VII	Hexahydro-2-oxo-4-(6,6-dicarboxylhexyl)-1-furo-3.4-imidazole	5	COOH —CH—CH COOH	480,000	13,400		(0.0010)		

		n	R						
VIII	Hexahydro-2-oxo-4-(4-hydroxybutyl)-1-furo-(3,4)-imidazole	4	—OH	2,600,000	152,000	>1,300,000	>600,000	>1,200,000	300,000
IX	Hexahydro-2-oxo-4-(4-mercaptobutyl)-1-furo-(3,4)-imidazole	4	—SH	600,000	1,400	>1,200,000	570,000	575,000	7,000
X	Hexahydro-2-oxo-4-(4-benzylmercapto-butyl)-1-furo-(3,4)-imidazole	4	—S—CH₂C₆H₅	740,000	9,300	850,000	74,000	100,000	1,900
XI	Sulfone of X	4	—SO₂—CH₂—C₆H₅	2,000,000	100,000	>1,000,000	>500,000	>2,000,000	125,000
XII	Oxybiotin sulfonic acid	4	—SO₃H	1,350,000	14,300	>1,800,000	1,320,000	>900,000	86,000
XIII	Hexahydro-2-oxo-4-(5-mercaptopentyl)-1-furo-(3,4)-imidazole	5	—SH	168,000	5,300	(0.0014)		500,000	12,500
XIV	Hexahydro-2-oxo-4-(5-benzylmercapto-pentyl)-1-furo-(3,4)-imidazole	5	—S—CH₂C₆H₅	765,000	8,900	1,000,000	125,000	48,000	760
XV	Sulfone of XIV	5	—SO₂—CH₂—C₆H₅		(0.012)		(0.0012)		(0.004)
XVI	Homooxybiotin sulfonic acid	5	—SO₃H				(0.014)		
XVII	cis-3,4-Diamino-2-tetrahydrofuran caproic acid sulfate			>2,000,000	>2,000,000	>2,000,000	>2,000,000	>2,000,000	>2,000,000

Eakin, and Williams medium (9) as modified by Hertz (10). **The organisms** were grown in 50 cc. Erlenmeyer flasks for 24 hours at 30°.

L. arabinosus—The stock culture was carried at 30° in agar stabs composed of the "synthetic" medium,[2] plus 0.1 per cent each of liver extract (Wilson, 1:20) and Difco yeast extract. To prepare an inoculum, the organisms from a stab were grown for 24 hours in 10 cc. of the synthetic medium plus 0.01 per cent of liver extract (Wilson, 1:20) and 0.05 per cent of Difco yeast extract. The resulting organisms were washed in normal saline and resuspended in 10 cc. of saline. 0.03 cc. of a 1:25 dilution in saline of this suspension was used to inoculate 10 cc. of the synthetic medium. A growth period of 72 hours at 30° was employed.

S. hemolyticus—As a stock culture, this organism was carried at 37° in semisolid veal heart infusion agar (0.2 per cent) containing 5 per cent defibrinated rabbit blood. The pH of this medium was adjusted to 7.6. The culture has been passed through mice at frequent intervals in order to maintain its virulence (minimum lethal dose for white mice two to six organisms). A 24 hour stock culture was plated on blood agar and incubated 24 hours at 37°. 5 cc. of the synthetic medium[3] were inoculated with the organisms from one colony. After incubation at 37° for 24 hours, the cells were washed with normal saline and resuspended in 5 cc. of saline. 0.03 cc. of this suspension was used to inoculate 5 cc. of the synthetic medium. A growth period of 24 hours at 37° was employed.

E. coli—The stock culture was kept at 37° on agar slants of Difco A. C. medium. To prepare an inoculum, the organisms were transferred from a 24 hour slant to a veal heart infusion broth containing 0.2 per cent glucose. After a growth period of 6 hours at 37°, the organisms were washed with normal saline and resuspended in 5 cc. of saline. 0.03 cc. of a 10^{-6} dilution of this suspension in saline was used to inoculate 5 cc. of MacLeod's medium (13). The culture was grown for 24 hours at 37°.

S. aureus—The stock culture was carried at 37° on agar slants of veal heart infusion broth. The procedure for the preparation of the inoculum was identical with that employed for *E. coli* with the exception that 0.03 cc. of the 5 cc. saline suspension of washed organisms was used directly without further dilution. The organisms were grown for 24 hours at 37° in 5 cc. of Wolf's medium (14) to which 12.5 mg. of glucose were added aseptically. A purified acid hydrolysate of casein (General Biochemicals, Inc.) was employed.

General Methods

The basal media utilized in studies of the inhibitory activity of the various compounds for *S. cerevisiae*, *L. arabinosus*, and *S. hemolyticus*

---

[2] The medium of Wright and Skeggs (11) plus 0.01 per cent of asparagine.

[3] Designated as Medium B in the paper by McIlroy, Axelrod, and Mellon (12).

(organisms which require an exogenous source of biotin for growth) were deficient in biotin and were supplemented with the following amounts of d-biotin or dl-oxybiotin: *S. cerevisiae*, 0.1 mμgm. of biotin or 0.4 mμgm. of oxybiotin per 10 cc.; *L. arabinosus*, 0.1 mμgm. of biotin or 0.2 mμgm. of oxybiotin per 10 cc.; and *S. hemolyticus*, 0.2 mμgm. of biotin or 0.4 mμgm. of oxybiotin per 5 cc. These amounts of biotin or oxybiotin produced approximately one-half maximum growth. Neither growth factor was added to the basal media employed for *E. coli* and *S. aureus* (organisms which do not require an exogenous source of biotin).

Compounds which exhibited antagonistic activities were added to the medium in increasing concentrations until growth was completely inhibited. The minimum quantity of antagonist necessary to inhibit growth completely was used to calculate the antibacterial index, *i.e.* the ratio of the molar concentration of the antagonist to that of d-biotin or dl-oxybiotin at which complete inhibition of growth results (15). Growth of *L. arabinosus* was determined by acidimetric titration. Turbidimetric measurements in the Evelyn photocolorimeter served to estimate growth of the remaining organisms. Because of their limited solubility in water, Compounds IX, X, XIII, and XIV were dissolved in aqueous ethanol. The amount of alcohol introduced was without effect upon growth of the microorganisms. All other compounds were tested in aqueous solutions. Solutions of each compound were sterilized by autoclaving in the various media.

Effect of dl-Homooxybiotin on S. hemolyticus Infections in Mice

These experiments were conducted with male, white mice weighing 25 to 30 gm. and receiving a diet of Purina chow. The virulent *S. hemolyticus* used in the experiments *in vitro* were injected intraperitoneally. dl-Homooxybiotin was given subcutaneously.

Experiment I—Eleven mice each received a single injection of 12,000 organisms. Five of these mice were given dl-homooxybiotin as follows: 20 mg. daily during the 2 days preceding the injection of the *S. hemolyticus*, 70 mg. (in seven equal doses) on the day of the injection, and 10 mg. the following day. All of the animals died within 84 hours after inoculation with the organisms.

Experiment II—Ten mice each received a single dose of ten organisms. Five of these mice were given 16 mg. of dl-homooxybiotin over a 4 day period beginning on the day before the injection of the *S. hemolyticus*. Four of the five animals in each group died within 72 hours after the introduction of the organism.

As controls, five mice which were not inoculated with *S. hemolyticus* received 16 mg. of dl-homooxybiotin in a manner identical with that of

the group treated above. No evidence of toxicity resulting from this amount of *dl*-homooxybiotin was noted.

Results

The inhibitory effects of Compounds I to XVII toward *S. cerevisiae*, *L. arabinosus*, and *S. hemolyticus* are shown in Table I. Compounds X and XIV were also tested for their antagonistic activities toward *E. coli* and *S. aureus*. Neither compound had any effect upon growth at a level of 600 γ per 5 cc. of medium.

The competitive nature of the antagonisms was evidenced by the fact that the antibacterial indices remained reasonably constant over a wide
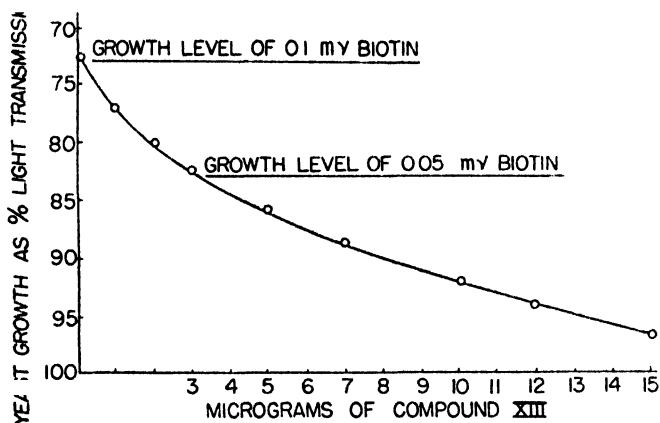
Fig. 1. Effect of varying amounts of *dl*-hexahydro-2-oxo-4-(5-mercaptopentyl)-1-furo-(3,4)-imidazole (XIII) on growth of *S. cerevisiae* in the presence of 0.1 mμgm. of biotin. The ordinate represents galvanometer readings on the Evelyn photocolorimeter. A blank reading (in the absence of added biotin) of 97 is obtained.

range of metabolite concentrations. The action of the inhibitors could be reversed completely by either biotin or oxybiotin.

In studies of biotin antagonists, two methods have been employed to obtain quantitative measurements of the inhibitory activities. Whereas we have expressed the inhibitory activity of our compounds in terms of antibacterial indices calculated at the level of *complete* growth inhibition, other workers (5, 16, 17) have calculated similar ratios at levels of *one-half* maximum inhibition. In order to compare our compounds with other antibiotins, it is necessary to determine the degree to which the inhibitory ratios are affected by the two different methods of calculation. A representative experiment with *S. cerevisiae* and Compound XIII is

presented in Fig. 1. It can be seen that the degree of growth inhibition is not a linear function of the amount of inhibitor present. Therefore, inhibition ratios calculated at the level of half maximum growth inhibition are smaller than those calculated at the level of complete inhibition. Similar results have been obtained in inhibition studies with the other oxybiotin derivatives as well as with the other organisms. It is apparent that our method of calculating inhibition ratios indicates a lower potency than does the other procedure. A similar conclusion can be reached by analysis of data presented by Dittmer and du Vigneaud (18).

<center>DISCUSSION</center>

In attempts to prepare new therapeutic agents, the biotin molecule has been structurally modified in a variety of ways (1–3, 16–22). Although the primary aim of these studies was the preparation of compounds biologically antagonistic to biotin, they have also yielded information emphasizing the high degree of biological specificity of this vitamin. Thus, its growth-promoting properties have usually been severely impaired by the structural modifications made to date. An outstanding exception is the case of oxybiotin, a compound in which the sulfur atom of biotin has been replaced by oxygen. The resulting oxygen analogue still possesses significant activity for a variety of microorganisms and higher animals. In this connection, desthiobiotin should also be mentioned. This compound possesses biotin-like activity for a number of organisms (23, 24). However, the fundamental difference in the mode of action of oxybiotin and desthiobiotin lies in the fact that the former compound is intrinsically active (25–28), while the latter is active because of its conversion into biotin (29–32).

None of the oxybiotin derivatives presented in this report possesses any significant biotin-like activity. Even such a minor modification as the introduction of a carboxyl group adjacent to the original carboxyl group in oxybiotin markedly impairs the growth-promoting activity. The inability of the microorganisms studied to decarboxylate this malonic acid derivative is, therefore, indicated. Further evidence for the high degree of specificity of the oxybiotin molecule is given by the fact that small changes in the length of the side chain (I to V) completely destroy biotin activity.

A logical step in attempts to develop biotin antagonists was to apply modifications to this molecule which in the case of other metabolites have resulted in antagonists. The replacement of carboxyl groups by sulfonic acid residues represents such a modification (33). Accordingly, oxybiotin sulfonic acid (XII) was prepared and found to possess antagonistic activity. The sulfonic acid analogue (XVI) of homooxybiotin (IV) was also syn-

thesized and, surprisingly enough, possessed slight growth-promoting activity for the three organisms. It is conceivable either that these organisms are capable of a limited synthesis of oxybiotin from a derivative containing the correct number of carbon atoms in the side chain or that traces of oxybiotin were formed during the synthesis of the compound. In the preparation of these sulfonic acids a number of intermediates were obtained (IX, X, XIII, and XIV) which were found to be even better antagonists than the desired sulfonic acids. The benzylthioether derivatives (X and XIV) had the disadvantage of being sparingly water-soluble and attempts were made to produce from them water-soluble derivatives with comparable antagonistic activity. The corresponding sulfones (XI and XV) were, therefore, prepared and were found to possess markedly decreased antagonistic activities. As in the case of the sulfonic acids, Compound XV, with a 5-carbon side chain, possessed a slight degree of growth-promoting potency. The explanation for this activity might be similar to the one offered above.

As previously mentioned, variations of the side chain length in the oxybiotin molecule completely destroy the biotin activity. The resulting compounds (I to V) possess varying degrees of antagonistic activity, which is greatest in homooxybiotin (Compound IV). Recently, a number of biotin homologues have been reported to possess a high degree of antagonistic activity. In particular, homobiotin was found to be the most potent antibiotin yet described (20). It is of interest that in both the biotin and the oxybiotin series the homologues, possessing a caproic acid side chain, were the most active compounds of this type. The antagonistic activity of homooxybiotin depends upon the presence of the intact imidazolidone ring. This is clearly evidenced by the fact that the cis-3,4-diamino-2-tetrahydrofurancaproic acid sulfate (XVII) was without activity.

We had previously indicated (1) that certain of the oxybiotin derivatives are more effective in antagonizing oxybiotin than biotin. This has also been shown to be the case with the additional oxybiotin derivatives presented in this paper. This situation holds true for each of the three organisms studied. Since all of the known biotin antagonists tested exhibit this differential effect (5, 22, 34, 35), one cannot attribute our results simply to the fact that oxybiotin derivatives were employed.

It is generally observed that organisms which are capable of growing in the absence of an exogenous source of a metabolite are resistant to the effects of the corresponding antimetabolite. In agreement with this observation we have found that E. coli and S. aureus, organisms which grow in the absence of added biotin, are not inhibited by the thioethers (X and XIV).

The ineffectiveness of homooxybiotin in combating *S. hemolyticus* infection in mice as presented in this paper poses an interesting question. Recent studies have amply demonstrated an interrelationship between biotin, aspartic acid, and certain fatty acids (36–41). Of particular interest in this connection is the observation that biotin antagonists lose their growth-inhibitory properties for microorganisms in the presence of aspartic acid and certain lipides (35, 42).[4] The inactivity of homooxybiotin in combating *S. hemolyticus* infection may be attributed to the fact that in the host animal conditions prevail which keep the organism supplied with aspartic acid and fatty acids, products which are normally synthesized in the organisms through the catalytic action of biotin.

SUMMARY

Varying degrees of antagonistic activity against biotin and oxybiotin have been found for a number of oxybiotin analogues with *Saccharomyces cerevisiae*, *Lactobacillus arabinosus*, and *Streptococcus hemolyticus*. Two of the more active antagonists were found to be inactive for organisms not requiring an exogenous source of biotin (*Escherichia coli* and *Staphylococcus aureus*). Homooxybiotin was without effect in an infection in mice with a strain of *Streptococcus hemolyticus*. A possible explanation for this inactivity has been offered.

BIBLIOGRAPHY

1. Hofmann, K., Chen, C., Bridgwater, A., and Axelrod, A. E., *J. Am. Chem. Soc.*, **69**, 191 (1947).
2. Hofmann, K., Bridgwater, A., and Axelrod, A. E., *J. Am. Chem. Soc.*, **69**, 1550 (1947).
3. Hofmann, K., Bridgwater, A., and Axelrod, A. E., *J. Am. Chem. Soc.*, **71**, 1253 (1949).
4. Axelrod, A. E., Purvis, S. E., and Hofmann, K., *J. Biol. Chem.*, **176**, 695 (1948).
5. Rubin, S. H., Flower, D., Rosen, F., and Drekter, L., *Arch. Biochem.*, **8**, 79 (1945).
6. Pilgrim, F. J., Axelrod, A. E., Winnick, T., and Hofmann, K., *Science*, **102**, 35 (1945).
7. Hofmann, K., *J. Am. Chem. Soc.*, **67**, 1459 (1945).
8. Mellon, R. R., and Cooper, F., *Proc. Soc. Exp. Biol. and Med.*, **38**, 158 (1938).
9. Snell, E. E., Eakin, R. E., and Williams, R. J., *J. Am. Chem. Soc.*, **62**, 175 (1940).
10. Hertz, R., *Proc. Soc. Exp. Biol. and Med.*, **52**, 15 (1943).
11. Wright, L. D., and Skeggs, H. R., *Proc. Soc. Exp. Biol. and Med.*, **56**, 95 (1944).
12. McIlroy, A. P., Axelrod, A. E., and Mellon, R. R., *J. Bact.*, **56**, 547 (1948).

[4] Axelrod, A. E., and Hofmann, K., unpublished observations.

13. MacLeod, C. M., *J. Exp. Med.*, **72**, 217 (1940).
14. Wolf, P. A., *J. Bact.*, **49**, 463 (1945).
15. McIlwain, H., *Brit. J. Exp. Path.*, **23**, 95 (1942).
16. Dittmer, K., and du Vigneaud, V., *Science*, **100**, 129 (1944).
17. English, J. P., Clapp, R. C., Cole, Q. P., Halverstadt, I. F., Lampen, J. O., and Roblin, R. O., Jr., *J. Am. Chem. Soc.*, **67**, 295 (1945).
18. Dittmer, K., and du Vigneaud, V., *J. Biol. Chem.*, **169**, 63 (1947).
19. Duschinsky, R., and Dolan, L. A., *J. Am. Chem. Soc.*, **68**, 2350 (1946).
20. Goldberg, M. W., Sternbach, L. H., Kaiser, S., Heineman, S. D., Scheiner, J., and Rubin, S. H., *Arch. Biochem.*, **14**, 480 (1947).
21. Brown, G. B., and du Vigneaud, V., *J. Biol. Chem.*, **163**, 761 (1946).
22. Duschinsky, R., and Rubin, S. H., *J. Am. Chem. Soc.*, **70**, 2546 (1948).
23. Melville, D. B., Dittmer, K., Brown, G B., and du Vigneaud, V., *Science*, **98**, 497 (1943).
24. Lilly, V. G., and Leonian, L. H., *Science*, **99**, 205 (1944).
25. Hofmann, K., and Winnick, T., *J. Biol. Chem.*, **160**, 449 (1945)
26. Axelrod, A. E., Flinn, B. C., and Hofmann, K., *J. Biol. Chem.*, **169**, 195 (1947).
27. McCoy, R. H., McKibben, J. N., Axelrod, A. E, and Hofmann, K., *J Biol. Chem.*, **176**, 1319, 1327 (1948).
28. Krueger, K. K., and Peterson, W. H., *J. Bact.*, **55**, 693 (1948).
29. Dittmer, K., Melville, D. B., and du Vigneaud, V., *Science*, **99**, 203 (1944)
30. Leonian, L. H., and Lilly, V. G., *J. Bact.*, **49**, 291 (1945)
31. Tatum, E. L., *J. Biol. Chem.*, **160**, 455 (1945).
32. Stokes, J. L., and Gunness, M., *J. Biol Chem.*, **157**, 121 (1945).
33. Woolley, D. W., *Science*, **100**, 579 (1944).
34. Axelrod, A. E., DeWoody, J., and Hofmann, K., *J. Biol. Chem.*, **163**, 771 (1946).
35. Scheiner, J. M., and Rubin, S. H., Abstracts, American Chemical Society, Washington, 114th meeting, 31C August (1948).
36. Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, **166**, 335 (1946).
37. Williams, W. L., Broquist, H. P., and Snell, E. E., *J. Biol. Chem.*, **170**, 619 (1947)
38. Stokes, J. L., Larsen, A., and Gunness, M., *J. Bact.*, **54**, 219 (1947).
39. Koser, S. A., Wright, M. H., and Dorfman, A., *Proc. Soc. Exp. Biol. and Med.*, **51**, 204 (1942).
40. Axelrod, A. E., Hofmann, K., and Daubert, B. F., *J. Biol. Chem.*, **169**, 761 (1947)
41. Potter, R. L., and Elvehjem, C. A., *J. Biol. Chem.*, **172**, 531 (1948).
42. Broquist, H. P., and Snell, E. E., *J. Biol. Chem.*, **173**, 435 (1948).

CRYSTALLINE GLYCEROPHOSPHATE DEHYDROGENASE FROM RABBIT MUSCLE

By TADEUSZ BARANOWSKI*

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis)

The following enzymatic reactions have been shown to occur in animal tissues and in yeast (1).

Fructose-1,6-diphosphate

Aldolase

D-Glyceraldehyde-3-phosphate $\longleftrightarrow$ dihydroxyacetone phosphate

Isomerase

Dehydrogenase

Dehydrogenase

D-Glyceric acid-1,3-diphosphate

L-α-Glycerophosphate
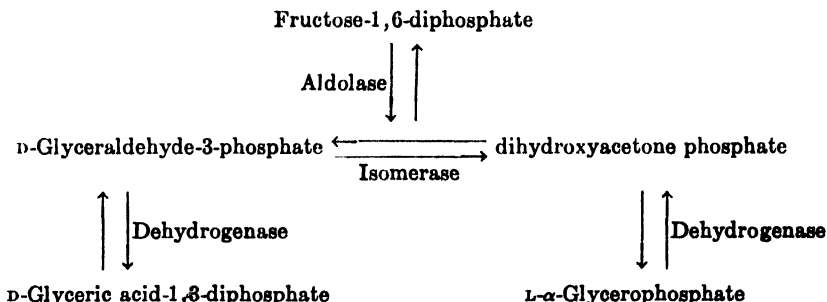
Aldolase and glyceraldehyde phosphate dehydrogenase from rabbit muscle have been obtained in crystalline form in this laboratory (2, 3). In the present paper is described the preparation of crystalline glycerophosphate dehydrogenase from rabbit muscle. This enzyme requires diphosphopyridine nucleotide (DPN) as coenzyme (4).

EXPERIMENTAL

Enzymatic activity was determined in an optical test, based on the measurement of reduced DPN at 340 mμ in the Beckman spectrophotometer. The reaction was carried out in phosphate or cysteine buffer at pH 7. Synthetic dihydroxyacetone phosphate (5) with reduced DPN or racemic α-glycerophosphate with DPN was used as substrate to measure the forward and backward reactions, respectively. The concentrations of reactants are given in Tables I and II. The protein concentration was measured optically at 280 mμ; the conversion factor for log I_0/I readings was obtained from protein determinations by the biuret method of Robinson and Hogden (6).

* Research Fellow of the World Health Organization. Present address, University Medical School, Wroclaw, Poland.

Isolation of Enzyme—As shown in the succeeding paper (7), recrystallized myogen A contains glycerophosphate dehydrogenase. It proved possible to separate the dehydrogenase from myogen A by fractional crystallization at an acid pH.[1] The optical test for enzyme activity and the ultraviolet absorption spectrum of the protein (see Fig. 1) were used as guides in the isolation. The following is a description of one preparation.

Myogen A bipyramids were prepared from 1 kilo of rabbit muscle as described in the succeeding paper (7). After several recrystallizations the
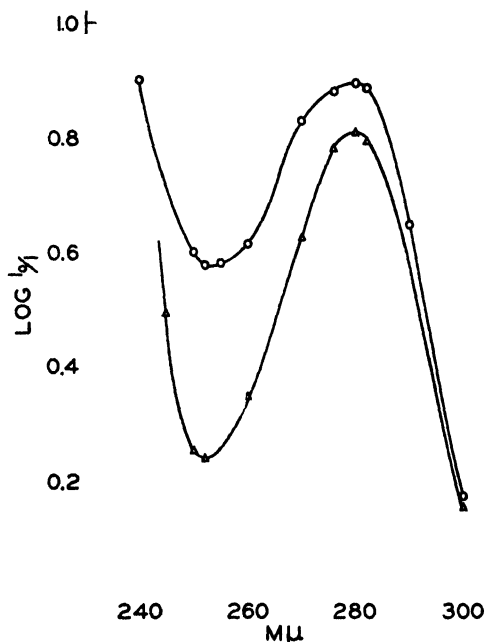
Fig. 1. Ultraviolet absorption curve of crystalline glycerophosphate dehydrogenase (O) and aldolase (△), each 1 mg. per cc., at pH 7.

aldolase activity was about 15 per cent of that found for a standard aldolase preparation, and the ultraviolet absorption in the region 250 mμ showed a greater density than that of aldolase. To a 1 per cent solution of the myogen A crystals was added ammonium sulfate until the necessary saturation (about 0.45) for crystallization was nearly reached. The pH was 5.5. A small crystalline precipitate which formed overnight in the

[1] The dehydrogenase is precipitated from rabbit muscle extract at pH 7.6 between 0.45 and 0.65 saturation of ammonium sulfate, but separation from other proteins is difficult. So far the enzyme has been prepared only from myogen A crystals.

cold room was removed. Then a few drops of saturated ammonium sulfate solution were added from time to time over a period of several weeks while the solution remained in the cold room. Successive fractions of crystals were collected and enzyme activity and ultraviolet absorption were determined for each fraction. The first crystalline fractions showed about 35 per cent of aldolase activity and only traces of glycerophosphate dehydrogenase activity. The middle fractions had lower aldolase activity, and finally bipyramids were obtained which showed high glycerophosphate dehydrogenase activity and only traces of aldolase activity. These were dissolved in a small amount of water, and solid ammonium sulfate was added until a distinct shimmer was observed upon stirring the solution.

On standing overnight in the cold two types of crystals settled out, needles and hexagonal plates. When the solution containing the crystals was brought to room temperature (about 28°), the hexagonal plates dissolved, while the less soluble needles, containing high glycerophosphate dehydrogenase activity, could be separated by centrifugation. The pH of the mother liquor was 5.45 and the saturation of ammonium sulfate 0.5. After recrystallization, rhombic plates, which looked like needles when standing on edge, were obtained (Fig. 2). Additional crystallizations yielded a preparation of constant enzymatic activity.

When the enzyme is precipitated from dilute aqueous solution in an amorphous form by ammonium sulfate, one finds that on standing in the cold room the precipitate is slowly converted to crystals. These crystals aggregate and assume the shape of whetstones. The specific enzymatic activity of this form of crystals was the same as that of the rhombic plates. Glyceraldehyde-3-phosphate dehydrogenase could not be detected in the crystals.

The glycerophosphate dehydrogenase crystals in half saturated ammonium sulfate were stable for several weeks in the cold room. In dilute solution the enzyme was unstable and lost 90 per cent of its activity in 24 hours at 0°. Activity was measured immediately after dissolving the crystals, because in the high dilution used in the test most of the activity was gone after 10 minutes. Dilution with cysteine did not prevent inactivation of the enzyme. Further work is necessary to study the conditions under which the enzyme can be stabilized. Since only small amounts of highly purified enzyme were obtained, only some of the properties of the enzyme could be explored at this time.

Turnover Number—An example of rate measurements with two different enzyme concentrations is given in Fig. 3. Based on the rate observed during the first 30 seconds, 100,000 gm. of protein catalyzed the reduction of 26,500 molecules of dihydroxyacetone phosphate per minute at 20°,

pH 7.0. At 30° the turnover number was about twice as great. Between 1 and 4 γ of enzyme per 3 cc., there was good proportionality between the rate of the reaction and the enzyme concentration.

The concentration of the enzyme in rabbit muscle was calculated from activity measurements in dialyzed muscle extracts to which iodoacetate had been added to inhibit glyceraldehyde phosphate dehydrogenase ac-

Fig. 2 Crystalline glycerophosphate dehydrogenase from rabbit muscle, × 260

tivity. On an average 0.015 gm. of enzyme per 100 gm. of muscle was found. About the same concentration was found in rabbit liver.

Equilibrium The equilibrium constant could not be determined accurately with dihydroxyacetone phosphate as substrate, because the equilibrium is shifted far to the side of glycerophosphate (see Table I). The experiment in Table I shows incidentally that the enzyme can be used for the quantitative determination of small amounts of dihydroxyacetone
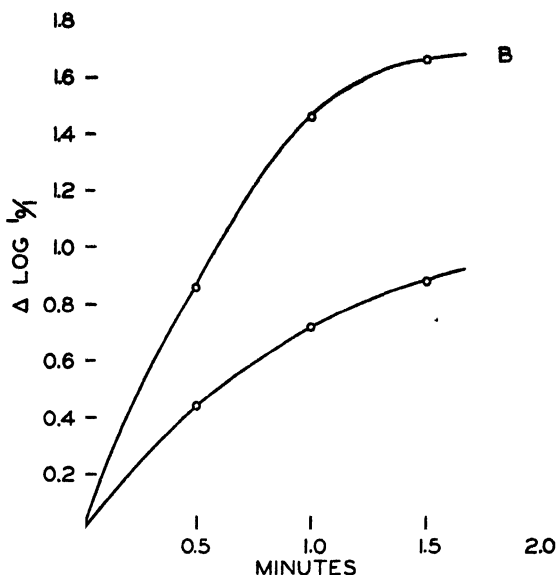
FIG. 3. Activity of α-glycerophosphate dehydrogenase. The reaction mixture contained, in moles per 3 cc., dihydroxyacetone phosphate 8.4×10^{-4}, and reduced DPN 1.1×10^{-7}. The pH of the cysteine buffer was 7 and the temperature 20°. At zero time 1.58 γ of crystalline enzyme were added to Curve A and 3.16 γ to Curve B.

TABLE I

Equilibrium with Dihydroxyacetone Phosphate

The reaction mixture contained, in moles per 3 cc., phosphate buffer 2×10^{-4}, pH 7, reduced DPN 9.0×10^{-7}, and dihydroxyacetone phosphate 5.6×10^{-7} (based on determination of alkali-labile P). Temperature 22°. Absorption cell 1 cm. At zero time 2.2 γ of crystalline enzyme were added.

Time	Log $\frac{I_0}{I}$	Δ log $\frac{I_0}{I}$
sec.		
0	1.90	
30	1.24	0.66
60	0.98	0.92
90	0.82	1.08
120	0.78	1.12
150	0.74	1.16
180	0.73	1.17
210	0.72	1.18*

* From $\beta = 1/cd \ln I_0/I = 1.45 \times 10^7$ moles of reduced DPN per cc., it may be calculated that $(2.3 \times 1.18 \times 3)/(1.45) \times 10^{-7} = 5.62 \times 10^{-7}$ mole of dihydroxyacetone disappeared. This corresponds closely to the amount which had been added.

phosphate. By approaching the equilibrium from the glycerophosphate side (Table II), K for the reaction, dihydroxyacetone phosphate + DPNH $\rightleftharpoons$ glycerophosphate + DPN, was found to be 1.4×10^4 at 22° at pH 7. K was calculated from the equation, $K = ([c - x] [g - x])/[x]^2$, where c and g are the initial concentrations of DPN and L-α-glycerophosphate, respectively, and x the concentration of reduced DPN at equilibrium.

Absorption Spectrum—The ultraviolet absorption spectrum of the enzyme (Fig. 1) is similar to that found for glyceraldehyde phosphate dehydrogenase (8), particularly in the region 250 to 260 mμ, where the adenine part of DPN shows strong absorption. Glyceraldehyde phosphate dehydrogenase has been shown to contain 1 DPN molecule per 5×10^4 gm. of protein (8). It seems possible that glycerophosphate de-

TABLE II

Equilibrium with Glycerophosphate

The reaction mixture contained, in moles per 3 cc., phosphate buffer 2×10^{-4}, pH 7, DPN 9.1×10^{-7}, and racemic α-glycerophosphate 1.8×10^{-5}. Temperature 22°. Absorption cell 1 cm. At zero time 2.2 γ of crystalline enzyme were added.

Time	Log $\frac{I_0}{I}$	Δ log $\frac{I_0}{I}$
min.		
0	0.055	
1	0.069	0.014
2	0.080	0.025
3	0.088	0.033
4	0.095	0.040
5	0.101	0.046
6	0.103	0.048
7	0.105	0.050
8	0.105	0.050

hydrogenase also crystallizes with DPN (or DPNH) as the prosthetic group, but this will have to await further analysis.

The absorption spectrum of aldolase is included for comparison. Aldolase does not contain DPN and this makes the determination of absorption at 250 mμ useful in the separation of the two enzymes.

SUMMARY

Myogen A crystals, prepared from rabbit muscle, were found to contain glycerophosphate dehydrogenase. The enzyme was separated from myogen A by fractional crystallization and isolated in the form of rhombic plates. After several recrystallizations the enzyme activity per mg. of protein remained constant. The activity was determined in an optical

test with reduced DPN and dihydroxyacetone phosphate as substrates. The turnover number per 100,000 gm. of protein was 26,500 at 20° and pH 7.0. The equilibrium constant could be determined with glycerophosphate as substrate and had the value of 1.4×10^4 at 22° at pH 7.0. Owing to the fact that the equilibrium of the reaction is so far to the side of glycerophosphate, the enzyme can be used for the quantitative determination of dihydroxyacetone phosphate. Rabbit muscle contains about 0.015 gm. of this protein per 100 gm. of tissue. A similar concentration was found in liver.

I wish to thank Dr. Carl F. Cori for his kind interest in this work.

BIBLIOGRAPHY

1. Meyerhof, O., *Ergeb. Physiol.*, **39**, 10 (1937).
2. Taylor, J. F., Green, A. A., and Cori, G. T., *J. Biol. Chem.*, **173**, 591 (1948).
3. Cori, G. T., Slein, M. W., and Cori, C. F., *J. Biol. Chem.*, **173**, 605 (1948).
4. von Euler, H., Adler, E., and Guenther, G., *Z. physiol. Chem.*, **249**, 1 (1937).
5. Kiessling, W., *Ber. chem. Ges.*, **76 A**, 869 (1934).
6. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **135**, 727 (1940).
7. Baranowski, T., and Niederland, T. R., *J. Biol. Chem.*, **180**, 543 (1949).
8. Taylor, J. F., Velick, S. F., Cori, G. T., Cori, C. F., and Slein, M. W., *J. Biol. Chem.*, **173**, 619 (1948).

ALDOLASE ACTIVITY OF MYOGEN A

By TADEUSZ BARANOWSKI* and T. RUDOLF NIEDERLAND†

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis)

The problem of the relation of myogen A to aldolase, both crystalline proteins isolated from rabbit muscle, has been touched upon in a previous paper from this laboratory (1). The relevant facts may be summarized as follows. Meyerhof and Beck (2) reported that myogen A, prepared by the method of Baranowski (3), showed 16 per cent of the aldolase activity found by Warburg and Christian (4) for their rat aldolase preparation. Taylor, Green, and Cori (1) isolated aldolase from both rabbit and rat muscle and described several crystalline modifications, one of which greatly resembled the characteristic myogen A crystals (hexagonal bipyramids). Although these preparations were electrophoretically homogeneous and showed the same specific activity when compared among themselves, they were only 40 per cent as active as Warburg and Christian's preparations. It will be shown in this paper that with an improved test the specific activity of aldolase, as prepared in this laboratory, was found to be the same as that reported by Warburg and Christian.

The fact that repeatedly recrystallized myogen A preparations never reach full aldolase activity has been confirmed and, as shown in this paper, is to be attributed in part to the presence of other proteins in the crystals. Previous work by Chrobak and Baranowski (5) has already indicated that myogen A is not homogeneous, since differences in birefringence and shape of crystals had been observed under identical conditions of crystallization.

EXPERIMENTAL

The first aim was to investigate the conditions under which maximal aldolase activity could be obtained in the optical test of Warburg and Christian (4). The pH optimum of aldolase was found to be at 7, with a barely noticeable falling off in rate at either pH 6.5 or 7.5. The latter pH was chosen for activity measurements. At pH 9 there was a decrease in the rate of aldolase activity of 30 per cent, when the optical test was

used. In a chemical test, with cyanide as the trapping agent for the phosphotrioses formed, an apparent pH optimum of 9 has been reported (6). This is probably due to the fact that trapping becomes incomplete at pH values below 9, thus simulating a decrease in the rate of aldolase activity. For the same reason the values obtained at pH 7.5 in the chemical test are lower than those obtained in the optical test.

In the optical test one adds hexose diphosphate, arsenate, aldolase, an excess of glyceraldehyde phosphate dehydrogenase, and diphosphopyridine nucleotide (DPN) and measures the rate of appearance of the reduced band of DPN at 340 mμ in the Beckman spectrophotometer. It is essential that the participating enzymes should be free of interfering enzymes, particularly of glycerophosphate dehydrogenase. As shown in the preceding paper (7), this enzyme has a high turnover number, and, since it catalyzes a reaction in which reduced DPN disappears, it interferes in the optical test for aldolase. Furthermore, there should be no triose phosphate isomerase present, since its equilibrium is far to the side of dihydroxyacetone phosphate.

In order to detect isomerase activity in the participating enzymes, hexose diphosphate was replaced by synthetic dihydroxyacetone phosphate (8) in the optical test.

The glyceraldehyde phosphate dehydrogenase from yeast was used in the test system in place of the corresponding muscle enzyme used by Taylor et al. (1); it is more stable than the muscle enzyme and can be used without cysteine. The yeast enzyme was prepared in crystalline form by Dr. E. G. Krebs by a slight modification of the method of Warburg and Christian (9). In the amounts used in the optical test (200 γ), the yeast enzyme showed no aldolase, triose phosphate isomerase, or glycerophosphate dehydrogenase activity. The latter was determined in an optical test with synthetic dihydroxyacetone phosphate and reduced cozymase as substrates, as described in the preceding paper (7). It was also possible to prepare aldolase essentially free of glycerophosphate dehydrogenase and triose phosphate isomerase.

In some cases it was desirable to determine aldolase activity first and then to see whether the aldolase preparation contained glycerophosphate dehydrogenase. In this case one measures first the rate of appearance of reduced DPN at 340 mμ with hexose diphosphate as substrate and then adds iodoacetate to stop the action of glyceraldehyde phosphate dehydrogenase. In the concentrations used (2.5 $\times$ 10^{-3} M) iodoacetate has no inhibitory effect on glycerophosphate dehydrogenase. The rate of disappearance of reduced DPN is then used as a measure of glycerophosphate dehydrogenase activity.

Turnover Number of Aldolase—The activity was determined at pH 7.5

which is close to the optimum. The concentrations of reactants, in a total volume of 3 cc., were the same as those used by Warburg and Christian (9).

It seemed desirable to determine aldolase activity not only by the method of Warburg and Christian, but also by another, independent test. Since the chemical test did not appear to be reliable, another optical test was devised in which use was made of crystalline glycerophosphate dehydrogenase (7). The system consists of hexose diphosphate, aldolase, reduced DPN, and an excess of glycerophosphate dehydrogenase.[1] The dihydroxyacetone phosphate formed in the aldolase reaction is converted to glycerophosphate, with the equilibrium very far to the side of the latter, so that the rate of disappearance of reduced DPN at 340 mμ can be taken as a measure of aldolase activity. There was good agreement in the specific activity of aldolase as determined by these two methods.

The protein concentration was measured optically at 280 mμ; $(-\log_{10}T)/0.91 = $ mg. of protein per cc. This conversion factor was based on an average of two gravimetric determinations of dialyzed aldolase dried at 80° over P_2O_5 in a high vacuum. A factor previously determined had the value of 0.81 (1); hence the turnover number calculated with this factor was about 10 per cent too low.

The preparation of aldolase in the form of needles at pH 7.5 followed the procedure described by Taylor, Green, and Cori (1). The first crystals were regularly 70 to 80 per cent pure aldolase. By recrystallization from a solution containing 0.05 M phosphate at pH 7.5 or 5.6 the purity could be raised. Recrystallization at the acid pH, in which case one obtains aldolase in the form of bipyramids, was more effective. Purification was continued until the activity could be increased no more by repeated crystallizations both at the acid and alkaline pH. Equally active aldolase could also be obtained by fractionation with acetone at low temperature, confirming Taylor *et al.* (1), but the yield was poor.

Microscopically the bipyramid crystals of aldolase (Fig. 1) were of only one type, as far as shape and birefringence are concerned. The electrophoretic patterns showed homogeneity over the pH range investigated (5.8 to 7.6) and the mobilities were close to those reported by Taylor *et al.* (1).

The turnover numbers for rabbit aldolase shown in Table 1 are higher than those previously reported from this laboratory and are well within the range of the values reported by Warburg and Christian (4) for rat aldolase. The reason for the higher turnover number now found has not

---

[1] Racker (10) made use of a similar system in the measurement of phosphofructokinase, the enzyme which converts fructose-6-phosphate to fructose diphosphate.

been explained satisfactorily. In particular, the glyceraldehyde phosphate dehydrogenase of muscle has been reexamined for the presence of triose isomerase and glycerophosphate dehydrogenase, with negative results. It is possible that the instability of this enzyme as compared to the yeast enzyme was a contributory factor, although recent measurements of aldolase activity have given identical results with either dehydrogenase as auxiliary enzyme.
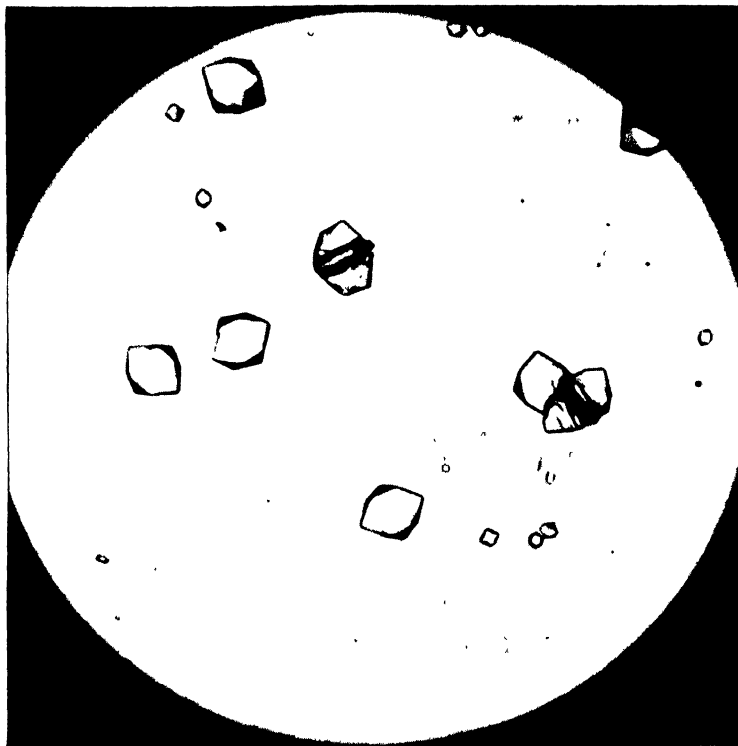
Fig 1 Rabbit aldolase crystallized from ammonium sulfate at pH 5.7 in the form of hexagonal bipyramids   × 260.

In Table II is given an example of the determination of the turnover number of aldolase with hexose diphosphate, glycerophosphate dehydrogenase, and reduced DPN. The value of 2550 at 23° is close to the value of 2150 reported by Warburg and Christian for rat aldolase at 20°.

Aldolase Activity of Myogen A—Table II shows that highly purified myogen A has a specific activity 0.35 of that of the standard aldolase preparation. Further crystallizations of this material at acid or alkaline pH did not change the activity.

As in the original procedure (3), 1 kilo of rabbit muscle was extracted twice with an equal volume of water. The protein fraction which precipitated at pH 5.6 between 0.4 and 0.6 saturation with ammonium sulfate

TABLE I

Activity of Crystalline Rabbit Muscle Aldolase

The test system was that described by Warburg and Christian (4).

Prepara-tion No.	Recrystallization	Amount of enzyme added per 3 cc.	$\Delta \log \frac{I_0}{I}$		Temper-ature	Turnover No.[*]
			After 1 min.	After 2 min.		
		γ			°C.	
1	3 times from (NH$_4$)$_2$SO$_4$ solution, pH 7.5	4.53	0.234	0.450	26	3690
2	3 times as above, in presence of phosphate buffer	7.53	0.340	0.670	26	3230
3	2 times at pH 7.6, 2 times at pH 5.6, in presence of phosphate buffer	7.48	0.390	0.778	28	3720
4	Acetone treatment	6.92	0.400	0.783	30	4140

* Calculated from the readings after 1 minute as moles of hexose diphosphate decomposed per 150,000 gm. of protein per minute at pH 7.5. $(2.3 \log I_0/I)/(1.45 \times 10^7)$ = moles of DPNH formed (or moles of hexose diphosphate decomposed) per

TABLE II

Specific Activity of Aldolase and of Myogen A

The test system contained, in moles per 3 cc., potassium phosphate buffer 1×10^{-4}, pH 7, fructose diphosphate 6×10^{-5}, reduced DPN 7×10^{-7}, and 10 γ of crystalline glycerophosphate dehydrogenase. Temperature 23°. The amounts of protein added at zero time were 4.25 γ of aldolase and 11.2 γ of myogen A.

Protein added	Time	$\log \frac{I_0}{I}$	$\Delta \log \frac{I_0}{I}$	Turnover No.[*]
	min.			
Aldolase	0	1.32		
	1	1.17	0.15	2550
	2	1.07	0.25	
Myogen A	0	1.33		
	1	1.19	0.14	900
	2	1.07	0.26	

* See foot-note to Table I.

was separated on a Büchner funnel, dissolved in 80 cc. of water, and heated for 10 minutes at 52°. The denatured protein was removed by filtration. The protein remaining in solution was salted out with ammonium sulfate

and separated on a Büchner funnel. After dissolving the precipitate in a small volume of water, ammonium sulfate was added to incipient turbidity. On keeping the material in a cold room, a semisolid gel formed in which there appeared after about 2 weeks the bipyramids of myogen A. Such preparations, even after repeated recrystallizations, showed an aldolase activity only 0.15 to 0.18 of that of the standard aldolase preparations.

The experiment in Table III shows that the myogen A crystals with 0.15 activity contain glycerophosphate dehydrogenase. Crystalline aldolase was added first in order to accumulate dihydroxyacetone phosphate and reduced cozymase. The action of the triose phosphate dehydrogenase was then stopped by the addition of iodoacetate. On addition of myogen A a rapid disappearance of reduced cozymase occurred, owing

TABLE III

Presence of Glycerophosphate Dehydrogenase in Myogen A

The reaction mixture contained, in moles per 3 cc., cysteine buffer 6×10^{-5}, DPN 9×10^{-6}, hexose diphosphate 4×10^{-4}, sodium arsenate 5×10^{-5}, and 200 γ of yeast triose phosphate dehydrogenase. The pH was 7.5 and the temperature 25°.

Additions	Time	Log $\frac{I_0}{I}$
	min.	
10 γ aldolase	0	0.00
Iodoacetate*	5	1.78
8 γ myogen A	15	1.86
	15.5	1.03
	16	0.79
	17	0.54

* Final concentration 2.5×10^{-3} M.

to the activity of the glycerophosphate dehydrogenase. After separation of this enzyme by fractional crystallization, as described in the preceding paper (7), one obtains myogen A crystals of 0.35 activity (Fig. 2). In Table IV it is shown that these crystals are free of glycerophosphate dehydrogenase activity.

Refractionation of myogen A of 0.35 activity failed to increase the activity except in a few preparations which yielded a small amount of less soluble bipyramids. These had an activity of 0.45 as compared to an activity of 1.0 for the aldolase standard. Occasionally an activity of 0.45 was obtained in the first crystalline myogen A fractions prepared from dialyzed muscle extracts. Muscle extracts prepared with 0.1 M KCl-K_2HPO_4 solution and then dialyzed against running tap water also yielded myogen A crystals of 0.45 to 0.5 activity.

It seems clear that with the methods used so far it has not been possible
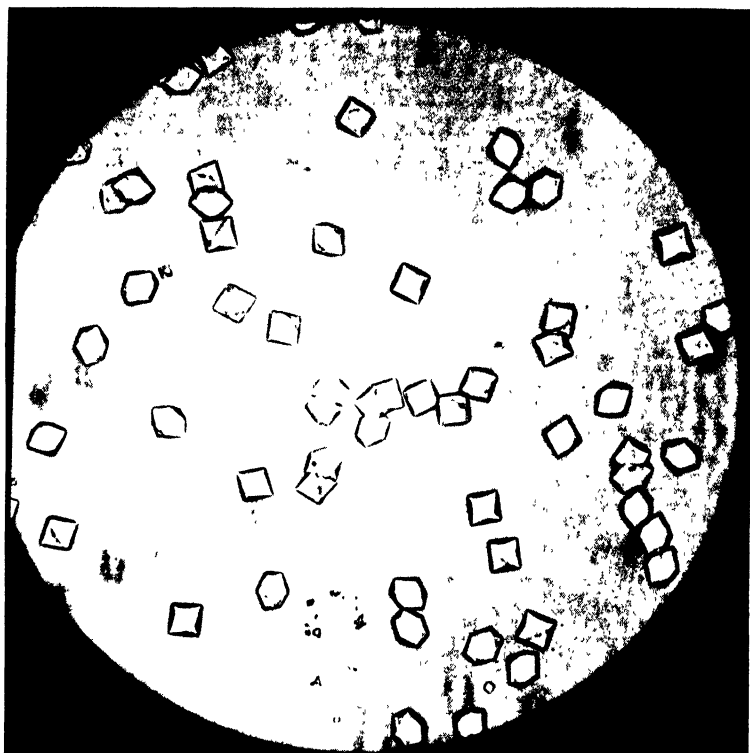
FIG 2 Crystalline myogen A, with an aldolase activity 35 per cent of that of pure aldolase   × 160

TABLE IV

Activity of Myogen A after Separation from Glycerophosphate Dehydrogenase

The reaction mixture was the same as in Table III.

	Time	Log $\frac{I_0}{I}$
	min	
15 5 γ myogen A	0	0 00
	1	0.28
	2	0.55
	3	0.83
Iodoacetate*	4	1.09
	6	1.15
	7	1.15
	8	1.15

* Final concentration 2.5×10^{-3} M.

to prepare pure aldolase from myogen A.   Addition of boiled muscle juice or of boiled aldolase failed to raise the activity of 0.35 active myogen

A. When myogen A and aldolase were mixed, the effects were additive; that is, myogen A did not inhibit aldolase activity.

Comparisons of different myogen A preparations with respect to electrophoretic mobility and other properties are shown in Table V. Birefringence was roughly estimated on crystals of considerable size, grown for this purpose. The ultraviolet absorption spectrum of myogen A of activity 0.15 differs from the other myogen A preparations and from aldolase in showing a strong absorption in the region 250 mμ. As discussed in the preceding paper (7), this is due to the presence of glycerophosphate dehydrogenase which has a different absorption spectrum.

TABLE V

Comparison of Myogen A Preparations with Different Aldolase Activities

Electrophoretic mobilities were determined in phosphate buffer, ionic strength 0 1, at 1.9°

Specific activity of myogen A (aldolase — 1 0)	Isoelectric point	Birefringence
0.15	5 9	+
0.35	6 0	+
0.45	6.2	+ +
1.0*	6.2	+ + +

* Aldolase.

DISCUSSION

That crystalline myogen A is not a homogeneous protein seems to be clearly established, even though electrophoresis and ultracentrifugation[2] reveal but one component. The typical bipyramids apparently represent mixtures or isomorphic crystals of several proteins, one of which, the glycerophosphate dehydrogenase, can be separated by fractional crystallization. The fact that these proteins migrate as one component in the electric field may be due to complex formation in solution, and it is probably significant in this respect that differences in the isoelectric point of different myogen A preparations have been detected.

Myogen A crystals, after separation from glycerophosphate dehydrogenase, show an activity which is 0.35 that of aldolase, and only in a few instances has it been possible to separate a small fraction with 0.45 to 0.5 activity. One important difference in the preparation of myogen A and of aldolase is that in the former case crystallization at acid pH requires 1 to 2 weeks, while in the latter case aldolase is crystallized overnight at alkaline pH. It is possible that an inactivation of aldolase occurs

during the rather long standing at acid pH, but in this case it is difficult to understand why the process stops at a definite level of aldolase activity, unless the formation of myogen A crystals depends on a definite ratio of active and inactive aldolase. Since other possibilities cannot be excluded, further work on the nature of myogen A seems necessary.

SUMMARY

1. The turnover number of crystalline aldolase from rabbit muscle has been redetermined with two independent and improved test systems. The value, in terms of moles of hexose diphosphate decomposed per 1.5×10^5 gm. of protein per minute at pH 7.5, is 2500 at 23° and 4200 at 30°. This is higher than was originally reported and agrees with the figure given by Warburg and Christian for rat muscle aldolase.

2. With an optical test, the pH optimum of aldolase was found to be at 7; at pH 9 the activity was 30 per cent lower. The apparent optimum reported previously at pH 9 in a chemical test with cyanide as the trapping agent was probably due to incomplete trapping of the phosphotrioses at pH values below 9.

3. Repeatedly recrystallized myogen A, prepared by the original method, showed an aldolase activity which was 15 per cent of that of a standard aldolase preparation. After complete separation from glycerophosphate dehydrogenase, the activity rose to 35 per cent, but could not be raised further by fractional crystallization.

4. Myogen A of 15 and 35 per cent aldolase activity was electrophoretically homogeneous between pH 5.8 and 7.6, but migrated faster at all pH values than aldolase. Ultracentrifugation also failed to reveal inhomogeneity. It may nevertheless be concluded that myogen A is not a homogeneous protein and is not identical with aldolase.

BIBLIOGRAPHY

1. Taylor, J. F., Green, A. A., and Cori, G. T., J. Biol. Chem., 173, 591 (1948).
2. Meyerhof, O., and Beck, L. V., J. Biol. Chem., 156, 109 (1944).
3. Baranowski, T., Z. physiol. Chem., 260, 43 (1939).
4. Warburg, O., and Christian, W., Biochem. Z., 314, 149 (1943).
5. Chrobak, L., and Baranowski, T., Compt. rend. Acad. sc. U. R. S. S., 28, 724 (1940).
6. Herbert, D., Gordon, H., Subrahmanyan, V., and Green, D. E., Biochem. J., 34, 1109 (1940).
7. Baranowski, T., J. Biol. Chem., 180, 535 (1949).
8. Kiessling, W., Ber. chem. Ges., 76 A, 869 (1934).
9. Warburg, O., and Christian, W., Biochem. Z., 303, 40
10. Racker, E., J. Biol. Chem., 167, 843 (1947).

CRYSTALLINE PROTEIN FROM COMMERCIAL BEANS (PHASEOLUS VULGARIS)

By JAQUES BOURDILLON

(From the Division of Laboratories and Research, New York State Department of Health, Albany)

A hitherto unrecognized crystalline substance having the properties of a protein has been isolated from commercial dried beans by the following method.

Commercial dried beans, variety Great Northern, were mechanically ground to a fine flour, of which 100 gm. were stirred with 400 ml. of N NaCl, followed by 500 ml. of 0.1 N HCl. The resulting pH was 2.8. The suspension was centrifuged for 30 minutes when some of the precipitate formed a layer on top of the supernatant solution instead of settling. However, this fraction adhered to the wall of the cup as the extract was decanted, and the opalescent fluid obtained was fairly clear. The pH was brought to 7.5 with about 40 ml. of N NaOH, and the new slight precipitate which formed was again removed by centrifugation. The pH was then lowered to 5.5 with about 5.0 ml. of N HCl, and 0.5 volume of ethyl alcohol was added at room temperature. The amorphous precipitate which formed in a few hours turned crystalline (or semicrystalline) upon standing overnight in the refrigerator. It was collected by centrifugation and washed once with cold 0.5 N NaCl to which 0.5 volume of alcohol had been added, and was dissolved in 200 ml. of N sodium acetate. The insoluble part was separated in the centrifuge and discarded. The supernatant was acidified to pII 5.5 with about 20 ml. of N HCl and the container placed in a 10 liter jar filled with water at 40°. Enough alcohol (about 0.7 volume) was added to produce a slight turbidity; crystallization proceeded as the water cooled to room temperature and the material was collected after 24 hours.

One recrystallization usually yielded entirely crystalline and completely soluble material. Occasionally, the solution of the first batch of crystals in sodium acetate yielded a suspension that could not be clarified by centrifugation. In such instances, recrystallization was carried out with the turbid solution, when the insoluble fraction was found to sediment easily the second time. The material was then crystallized a third time. The crystals were octahedra, sometimes elongated, most often in the shape of bipyramids (Fig. 1, left). If the wall of the container was scratched slightly, they formed against it as large jagged clusters as much as 2 mm. in length and strongly anisotropic (Fig. 1, right). The crystalline material

was dialyzed against distilled water and dried *in vacuo* to a white fluffy powder. The yield was 4 gm. from 100 gm. of flour.

The protein was sparingly soluble in cold distilled water, but was readily soluble at 50°. The warm solutions were water-clear with a slight blue opalescence, and remained clear on cooling. These were actually super-saturated solutions which gave precipitates upon addition of a trace of salt (acetate buffer of pH 5.5 to the extent of 0.001 M). The precipitate redissolved completely when the molarity was raised to 0.05. With ammonium sulfate, precipitation began only above 80 per cent saturation. On
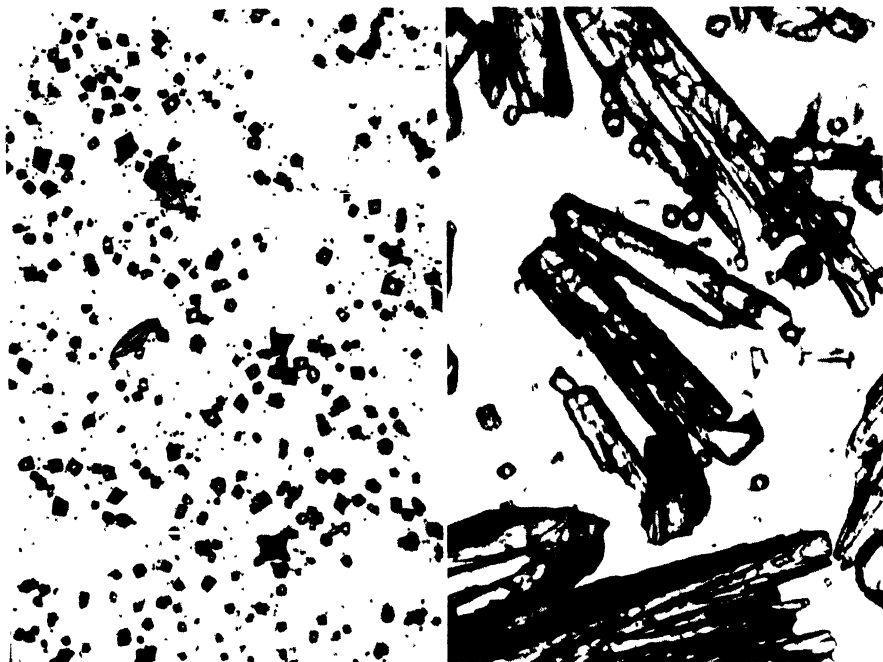
Fig. 1. Recrystallized bean protein. Magnification, × 45

the addition of alcohol, acetone, or dioxane at room temperature, precipitation began at about 30 per cent when the salt molarity was 1.0, and was more rapid at lower temperatures. The crystals could be dried with 95 per cent alcohol without loss of their shape or their solubility in water. The protein was denatured at boiling temperature. At pH 5.5, in the presence of salt, it began to precipitate at 95°, while in the absence of salt it could be boiled and cooled without apparent alteration, and was precipitated only when salt was added. It was irreversibly precipitated by trichloro-acetic acid.

Analysis of the recrystallised material gave the following information: carbon (Van Slyke and Folch) 50.4 per cent; nitrogen (Kjeldahl) 15.3 per cent; carboxyl nitrogen (Van Slyke), not detected; phosphorus, not detected; total sulfur (Carius) 0.12 per cent; sulfur as SH, not detected; carbohydrate (as glucose, Molisch), about 2 per cent; tyrosine-tryptophan (Folin), +; tryptophan (Hopkins-Cole), +; ash 0.2 per cent.

The sulfur content is particularly low and indicates a minimum molecular weight of about 27,000.

The acid- and base-binding power of the protein was measured in 1.2 per cent concentration and 0.5 M KCl with the help of the glass electrode (Table I). The figures given represent the difference between the total amount of HCl or KOH added and that accounting for the pH of the solution. At pH 6.5, 7.8, and 9.0, the figures are estimates because of drift in the pH readings, which, however, were not sufficient to affect their order

TABLE I

Acid- and Base-Binding of Bean Protein in 0.5 M KCl, Measured with Glass Electrode

pH	Acid bound per gm.	pH	Alkali bound per gm.
	mM		*mM*
1.54	0.89	5.5	0
1.70	0.74	6.5	0.10
1.92	0.55	7.8	0.15
2.15	0.54	9.0	0.19
3.16	0.39	10.4	0.38
4.08	0.206	11.0	0.54
4.61	0.105	11.3	0.89

of magnitude. These data establish the nature of the substance as an ampholyte with an isoionic point near pH 5.5, which was also that of minimum solubility and the optimum point for crystallisation. Comparison with the data of Cannan and his colleagues shows that the protein is a poor buffer, binding only one-third to one-half as much acid or base as egg albumin (1) or β-lactoglobulin (2), except at extreme pH values.

The molecular weight of the protein was determined by the author's osmotic pressure method (3) in 0.96 per cent concentration in 0.2 M phosphate buffer, pH 7.2, and found to be 171,000. This is 6 times the minimum weight estimated from the sulfur content.

The protein was only slightly susceptible to peptic hydrolysis. At pH 1.5, after 18 hours at room temperature in the presence of 0.01 its weight of pepsin, it was transformed into a substance insoluble over the pH range of 1 to 11, and yielded but little non-protein nitrogen. At pH 3.5, the same treatment left the protein unaffected and able to crystallise as

readily as before. The protein formed compounds with pepsin similar to those which pepsin forms with edestin (4) and split diphtheria antitoxin (5). The complex was least soluble at pH 4 and contained about 2 molecules of crystalline pepsin to 1 of vegetable protein. As with split antitoxin, there was evidence that the composition of the complex varied with the pH; attempts to isolate it in crystalline form were unsuccessful.

The protein may be characterized as an albumin because of its high solubility in the presence of ammonium sulfate, or as a globulin because of its insolubility in cold water at the isoelectric point. The yield being 4 gm. from 100 gm. of flour, and the flour containing 3.79 per cent nitrogen, this protein represents at least 16 per cent of all the nitrogenous material present.

According to Jones and his associates (6) seeds of all members of the genus *Phaseolus* contain an α- and β-globulin, the former precipitating with ammonium sulfate at less than half saturation and having a sulfur content of 1.0 to 1.5 per cent, the latter more soluble in the presence of ammonium sulfate and with a sulfur content of 0.3 to 0.4 per cent (6). A substance more like an albumin was obtained in denatured form from Lima beans, with a sulfur content of 1.15 per cent (7). Osborne (8) identified as phaselin an albumin-like material containing 0.49 per cent sulfur. The lack of crystallinity of these substances and the absence of clear physical characterization preclude comparison with the present results, and the conclusion seems justified that the protein described in this paper is a newly recognized entity.

SUMMARY

Commercial dried beans (*Phaseolus vulgaris*) yielded a crystalline protein with a molecular weight (by osmotic pressure) of 171,000 and an isoionic point near pH 5.5. At this pH, the substance is soluble in warm distilled water and precipitates only above 80 per cent saturation with ammonium sulfate. Its sulfur content is very low. The protein represents at least 16 per cent of the total nitrogenous material of the seed.

BIBLIOGRAPHY

1. Cannan, R. K., Kibrick, A., and Palmer, A. H., *Ann. New York Acad. Sc.*, **41**, 243 (1941).
2. Cannan, R. K., Palmer, A. H., and Kibrick, A. C., *J. Biol. Chem.*, **142**, 803 (1942).
3. Bourdillon, J., *J. Biol. Chem.*, **127**, 617 (1939).
4. Northrop, J. N., *J. Gen. Physiol.*, **17**, 165 (1933).
5. Bourdillon, J., *Arch. Biochem.*, **8**, 37 (1945).
6. Jones, D. B., Gersdorff, C. E. F., and Phillips, S., *J. Biol. Chem.*, **122**, 745 (1937-38).
7. Jones, D. B., Gersdorff, C. E. F., Johns, C. O., and Finks, A. J., *J. Biol. Chem.*, **53**, 231 (1922).
8. Osborne, T. B., *J. Am. Chem. Soc.*, **16**, 703, 757 (1894).

MEASUREMENT OF CARBONIC ANHYDRASE ACTIVITY OF BLOOD AT BODY TEMPERATURE*

By M. D. ALTSCHULE AND H. D. LEWIS

WITH THE TECHNICAL ASSISTANCE OF T. KISIL

(From the Medical Service and Medical Research Laboratories, Beth Israel Hospital, and the Department of Medicine, Harvard Medical School, Boston)

Most measurements of blood carbonic anhydrase activity recorded in the literature have been made by the method of Meldrum and Roughton (1) or some modification of it. These procedures are carried out in a cold water bath at 10° or below. This temperature is disadvantageous technically, and, moreover, affords no direct information as to the activity of the enzyme at mammalian body temperature; it is well known that low temperatures exaggerate the apparent activity of the enzyme markedly (1–4). In addition, the enzyme, under some circumstances, may be partially inactivated at low temperature (2, 5, 6). Methods based on that of Meldrum and Roughton (1) are unsatisfactory, also, in that violent shaking is required to counteract the retarding effect of cold on the diffusion of the evolved gas; variations in the rate and angle of rotation of the shaker used give rise to apparent variations in enzyme activity. Differences in size and shape of the vessels used also cause large differences in the values obtained (7). Mitchell, Pozzani, and Fessenden (8) have shown also that calculation of enzyme activity by means of the formula of Meldrum and Roughton (1) is not valid mathematically and yields misleadingly high values of enzyme activity. Mitchell *et al.* found that the catalyzed and the uncatalyzed reactions used in the estimation of carbonic anhydrase activity were unimolecular in character; accordingly they introduced the utilization of the principle of Guggenheim (9), based on velocity constants, in the measurement of activity of the enzyme. The present method is a modification of the procedure of Mitchell *et al.* (8), designed to estimate carbonic anhydrase activity in blood at 37°. It is to be noted that the velocity constant of the reaction is only one of several variables in the method; so that the values obtained are related to, but not the same as, the velocity constant.

Method

The basis for the determination of carbonic anhydrase activity is the usual one of its catalysis of the decomposition of carbonic acid at approximately pH 6.8.

Reagents—

Phosphate buffer. Solutions of 0.2 M monopotassium acid phosphate (KH_2PO_4) and 0.2 M disodium phosphate (Na_2HPO_4) are prepared and stored in separate containers. Just before use equal amounts of these solutions are mixed.

Bicarbonate solution. 0.2 M sodium bicarbonate is dissolved in 0.02 M sodium hydroxide. This solution is sealed in 20 ml. glass ampules, one of which is broken just before use, and the alkali mixed with an exactly equal quantity of distilled water.

Apparatus—The manometric method of Meldrum and Roughton (1) has been adapted to the use of standard Warburg vessels, manometers, constant temperature water bath, and shaking apparatus, as suggested by Stadie *et al.* (10). The water bath is maintained at 37°, and the vessels are shaken through an arc of about 30°, making 63 complete oscillations per minute. The rate of evolution of carbon dioxide is estimated from changes in pressure as read in the manometer; this rate is so rapid under the conditions of the method that it is directly proportional to the differences in manometric readings (11), and variations in the volumes of carefully made Warburg flasks and manometers are far too small to be of significance as sources of error in the present method.

Determination of Enzyme Activity—Heparinized venous blood is used; this is laked and diluted with distilled water in volumetric flasks and brought to the dilutions (in ml.) usually used, 1:20, 1:50, and 1:200. 0.5 ml. of diluted blood is placed in the Warburg flask; two glass beads and 1.0 ml. of phosphate buffer are added. 1 ml. of bicarbonate solution is added to the side arm. For each day's determinations a thermobarometer flask is prepared containing 2.5 ml. of distilled water.

The reaction vessel containing the reagents and enzyme is attached to its manometer and placed in the water bath for 10 minutes of equilibration. It is then removed, tilted gently three times to mix the solutions and to rinse the side arm, and returned to the water bath. The shaker is then started. The manometer stop-cock is left open for varying intervals of shaking, allowing the carbon dioxide rapidly evolved during the initial part of the reaction to escape, since the measurement of the rapid initial changes is inaccurate. Control reactions are allowed to proceed with the manometer stop-cock open for 90 seconds, catalyzed reactions with 1:200 blood for 50 seconds, catalyzed reactions with 1:50 blood for 40 seconds, and catalyzed reactions with 1:20 blood for 30 seconds. After the stated interval, the shaker is stopped, and the manometer is rapidly adjusted to the zero mark, the manometer stop-cock closed, and shaker and stop-cock are started again. Thereafter, the shaker is stopped to permit manometer readings every 30 seconds for up to 7 minutes. Thermobarom-

eter readings are made at the same time and the appropriate corrections applied to the readings of the reaction manometer. The recently introduced circular Warburg apparatus makes stopping of the shaker unnecessary.

The control, or uncatalyzed, reaction is observed in a similar manner, except that in place of blood 0.5 ml. of a 1:200 dilution of plasma in distilled water is used. This control solution was found to give smoother and more consistent curves than when distilled water alone was used as diluent. Neither enhancing nor inhibitory effects on enzyme activity
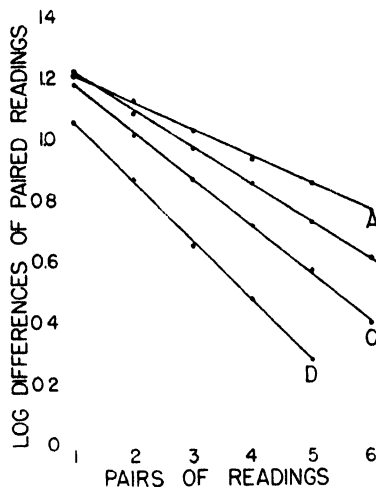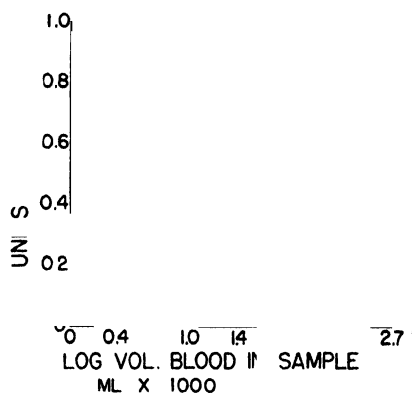
FIG. 1. Logarithms of differences of paired readings of rate of elaboration of carbon dioxide. Curve A, control solution; Curves B, C, and D, 0.5 ml. of blood diluted 1:200, 1:50, and 1:20, respectively.

FIG. 2. Calculation of value for undiluted blood by extrapolation from values found for three dilutions.

were found when this or even higher concentrations of human plasma were used.

All estimations were made in duplicate or triplicate, closely agreeing determinations being averaged.

Calculations—Calculation of activity of each sample is made from the data according to the method suggested by Mitchell, Pozzani, and Fessenden (8), and activity is expressed in terms of K, related to unimolecular velocity constants.

Pressure readings are taken at 30 second intervals as long as measurement of the rate of evolution of gas is accurate and these are corrected

for thermobarometer changes. These corrected readings are then paired, the differences taken, and the logarithm of these differences plotted against time (9) (Fig. 1). As the reaction proceeds, the back reaction of hydration becomes increasingly significant and some of the terminal points may have to be discarded in order to secure a straight line. The slope of the straight line thus obtained is multiplied by 2.303 to give the value K related to the velocity constant for the reaction.

Enzyme activity is expressed as the difference (K) between K_0, the value related to the velocity constant of the control reaction, and K_c, that related to the velocity constant of the reaction in the presence of the enzyme as follows:

$$K = K_c - K_0$$

Values of K for each dilution are then plotted against the logarithm of the amount of blood used in the experiment (Fig. 2) and a straight

TABLE I

Effect of Freezing and Thawing on 1:200 Dilution of Human Blood

Experiment No	K^* before	K^* immediately after
1	0.20	0.11
2	0.21	0.06
3	0.21	0.14
4	0.13	0.14
5	0.16	0.11

$*K$ = activity.

line drawn. By extending this line to zero dilution, an extrapolated value for 0.5 ml. of undiluted blood is obtained, this value being then multiplied by 2 to give the enzyme activity for 1.0 ml. of whole blood.

DISCUSSION

It is evident that values for blood carbonic anhydrase activity obtained at body temperature are more significant for mammalian physiology than those obtained at 0–10°, particularly since working in the cold exaggerates the apparent activity of the enzyme (1–4). Moreover, extreme cold partially inactivates the enzyme, as was shown when attempts were made in the present study to resort to freezing and thawing in order to avoid the need of laking the blood in water. When the blood was frozen at $-5°$ and thawed three times, the activity of the enzyme was markedly reduced (Table I).

The findings of Meldrum and Roughton (1) showed an absence of linear relation between concentration of the enzyme and its apparent activity

when blood is tested. The data of van Goor (12), Lambie (13), Philpot and Philpot (14), and of the present study are in accord. It was found here that when blood was used in high dilution, *i.e.* 1:200, the calculated activity per ml. of whole blood found was greater than in lower dilution, *i.e.* 1:20 or 1:50. However, when the logarithm of the amount of blood used was plotted against the activity found, a straight line could be drawn. Extrapolating this line to zero dilution gave the activity of 0.5 ml. of undiluted blood. The advantage of using the activity of undiluted blood for comparison of one specimen with another is apparent; it eliminates errors which might arise through the use of variously diluted bloods in the case of anemia or polycythemia. It might be considered that extrapolation introduces significant errors, as indeed it does; such errors are smaller, however, than those caused by basing conclusions on observations made only at high dilutions, as is the rule in methods previously described.

TABLE II

Observations in Forty-Two Normal Subjects

	Range	Average
Hematocrit, % *erythrocytes*.	38.0 −56.0	45.2
Hemoglobin, *gm. per ml. blood*	0.133– 0.199	0.138
Erythrocytes, *billions per ml. blood*	3.82 – 5.93	4.87
Carbonic anhydrase, *units per ml. blood*	1.2 – 2.6	1.8
" " " " " *erythrocytes*	2.6 – 5.8	4.05
" " " " *gm. hemoglobin*	8 −17	13
" " " " *billion erythrocytes*	0.25 – 0.56	0.37

The need for violent shaking to insure adequate diffusion of the gas evolved is obviated by the use of a high bath temperature.

By the present method and with the calculations used here the carbonic anhydrase activity is 1.2 to 2.6 (mean 1.8) units per ml. of normal blood (Table II; Fig. 3); *i.e.*, the value related to the velocity constant of the reaction which occurs when 1 ml. of blood causes the release of carbon dioxide from the reaction mixture is 1.2 to 2.6 times greater than that of the uncatalyzed reaction. Since all of the enzyme is within the red blood cells, its concentration might be expressed as 2.6 to 5.8 units per ml. of erythrocytes (average 4.05) (Table II). In agreement with the findings of Booth (15) no evidence of an inactivator was found in human plasma. The activity of the enzyme found here is almost certainly somewhat lower than the activity of the enzyme as it exists in the erythrocytes of the body, for the reaction mixture has a final pH of 6.90, a level at which carbonic anhydrase activity is decreased to some extent.

Data on the influence of various ions upon carbonic anhydrase activity have been reviewed by Roughton and Booth (16), who made extensive additional observations; it is clear that the chemical environment in which the enzyme is placed in the method here described is so markedly different from that which obtains in the red blood cell as to make it impossible to estimate the quantitative effects of the action of carbonic anhydrase in intact blood from results obtained with methods of the type described here. The work of Mitchell *et al.* (8) removed some of the mathematical invalidities from the earlier methods. The present method removes invalidities due to low temperature and to the lack of linear relation between enzyme concentration and its activity. However, conclusions as to the amount of acceleration caused by carbonic anhydrase in the hydration of carbon dioxide in the blood *in vivo* cannot be made from data now available.
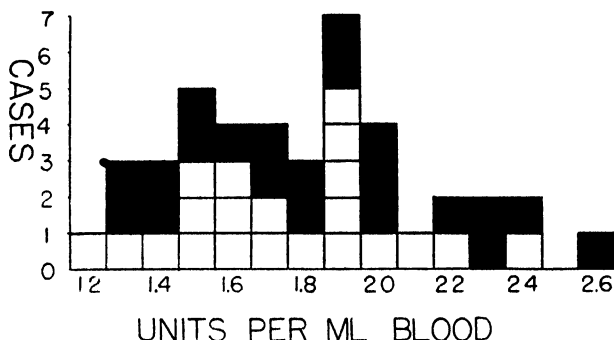
FIG. 3. Carbonic anhydrase activity of whole blood in forty-two normal subjects. The solid squares indicate men; the clear squares women.

SUMMARY

A method, in which a value related to unimolecular velocity constants is employed, has been devised for measuring carbonic anhydrase activity of blood at body temperature with standard Warburg apparatus. It has the advantages of eliminating some errors inherent in earlier methods, such as those consequent to low temperatures, lack of linear relation between enzyme concentration and activity, and mathematical invalidity of calculations used. On the other hand it affords no true indication of activity of the enzyme *in vitro* because of the differences in pH and in ionic constitution of the media used as against the interior of erythrocytes in blood.

BIBLIOGRAPHY

1. Meldrum, N. V., and Roughton, F. J. W., *J. Physiol.*, **80**, 113 (1933).
2. Main, E. R., and Locke, A., *J. Biol. Chem.*, **140**, 909 (1941).

3. Keilin, D., and Mann, T., *Biochem. J.*, **34**, 1163 (1940).
4. Roughton, F. J. W., *J. Physiol.*, **107**, 12 P (1948).
5. Kiese, M., and Hastings, A. B., *J. Biol. Chem.*, **132**, 267 (1940).
6. Roughton, F. J. W., and Booth, V. H., *Biochem. J.*, **40**, 309 (1946).
7. Lawrence, W. J., *Med. J. Australia*, **1**, 587 (1947).
8. Mitchell, C. A., Pozzani, U. C., and Fessenden, R. W., *J. Biol. Chem.*, **160**, 283 (1945).
9. Guggenheim, E. A., *Phil. Mag.*, **2**, 538 (1926).
10. Stadie, W. C., Riggs, B. C., and Haugaard, N., *J. Biol. Chem.*, **161**, 175 (1945).
11. Dixon, M., Manometric methods, New York, 2nd edition, 11–14 (1943).
12. van Goor, H., Doctorate thesis, Groningen (1934).
13. Lambie, C. G., *Edinburgh Med. J.*, **45**, 373 (1938).
14. Philpot, F. J., and Philpot, J. St. L., *Biochem. J.*, **30**, 2191 (1936).
15. Booth, V. H., *J. Physiol.*, **91**, 474 (1938).
16. Roughton, F. J. W., and Booth, V. H., *Biochem. J.*, **40**, 319 (1946).

COMPARISON OF RESPONSE OF SUCCINIC DEHYDROGENASE AND SUCCINOXIDASE SYSTEMS TO ORGANIC MERCURIAL AND THIOL COMPOUNDS

BY CORNELIUS KREKE, SISTER M. HONORA KROGER, AND ELTON S. COOK

(From the Division of Chemistry and Biochemistry, Institutum Divi Thomae, Cincinnati)

Our earlier observations (1), extended in the present report, had indicated that the aerobic succinoxidase system was more sensitive to phenylmercuric nitrate than the anaerobic succinic dehydrogenase system. Slater in a preliminary communication (2) called attention to a similar difference in sensitivity to p-chloromercuribenzoic acid. In studies of protection against mercurial inhibition by thiol compounds we noted the difference in response of the anaerobic and aerobic systems as well as the depressing effects of the thiols themselves on the aerobic system. Slater (3) and Barron *et al.* (4) meanwhile have reported similar effects for BAL and other dithiols. In addition we have noted an enhanced toxicity of cysteine and mercurial towards the aerobic system under certain conditions. Part of our results, although obtained independently and often with different compounds, are thus essentially confirmatory and will be presented only briefly.

EXPERIMENTAL

Succinic Dehydrogenase System—The succinic dehydrogenase activity was determined by the Thunberg methylene blue technique as described by Green and Dixon (5). Control tubes contained 0.9 ml. of a methylene blue solution (made from 8.0 ml. of 1:5000 methylene blue plus 6.0 ml. of 0.2 M phosphate buffer, pH 7.2), 0.1 ml. of 0.01 M sodium succinate, 1.0 ml. of phosphate buffer (pH 7.2), and 1.0 ml. of enzyme-containing tissue extract. The tissue extract was placed in the hollow stopper and was tipped into the substrate-methylene blue solution after evacuation (5 minutes) and equilibration (10 minutes). The temperature of the water bath was 37.5°. In the experimental tubes, 0.5 ml. of the mercurial compound solution replaced 0.5 ml. of buffer, and in experiments for testing protection 0.5 ml. of cysteine hydrochloride solution replaced a further 0.5 ml. of the phosphate buffer. These solutions were prepared in phosphate buffer at pH 7.2.

Succinoxidase System—The activity of the succinoxidase system was measured manometrically by the method of Schneider and Potter (6). The center well of the flasks contained 0.2 ml. of 2 N sodium hydroxide. The large chamber contained 0.4 ml. of 10^{-4} M cytochrome c (referred to a molecular weight of 16,500), 0.3 ml. of 0.5 M sodium succinate (recrystallized), 0.3 ml. of 4×10^{-3} M calcium chloride, 0.3 ml. of 4×10^{-3} M aluminum chloride, and 0.4 ml. of enzyme-containing tissue extract. Control flasks contained 1.3 ml. of phosphate buffer, pH 7.4. In the experimental flasks, materials to be tested for their effect on the enzyme activity were added to the contents of the flasks in place of phosphate buffer. Glass-distilled water was used in making solutions of the chemicals.

The enzyme for both systems was prepared from the hearts of 6 to 12 month-old albino rats and used as crude tissue extracts. The heart was ground in a mortar with sand and for every gm. of tissue 10 ml. of phosphate buffer were added. For the succinoxidase studies the tissue suspension was filtered through cheese-cloth and 2.0 ml. were diluted with 4.6 ml. of glass-distilled water. For the succinic dehydrogenase studies the tissue suspension was centrifuged and used without dilution.

Cytochrome c was prepared from beef hearts according to the procedure described by Keilin and Hartree (7), except that the enzyme was dialyzed against glass-distilled water instead of 1 per cent sodium chloride solution.

The basic phenylmercuric nitrate and phenylmercuric hydroxide were obtained from The Hamilton Laboratories, Inc. p-Chloromercuribenzoic acid was supplied by the Schaefer Laboratories. L-Cysteine hydrochloride was obtained from General Biochemicals, Inc. Thioglycolic acid was a product of the Fisher Scientific Company. Stock solutions of the materials were made in distilled water. L-Cystine, obtained from the Eastman Kodak Company, was dissolved in hydrochloric acid and the pH of the solution was adjusted to 7.4 with 2 N sodium hydroxide to give a concentration of 5×10^{-3} M. Dilutions of all stock solutions were made in phosphate buffer (pH 7.2 for the dehydrogenase and pH 7.4 for the oxidase system). All concentrations of these materials recorded in this paper refer to final concentrations in the flasks or tubes.

Results

Succinic Dehydrogenase System—The depression of the anaerobic system by phenylmercuric nitrate, phenylmercuric hydroxide, and p-chloromercuribenzoic acid is illustrated by the data in Table I, which gives the concentrations of the mercurials causing approximately 50 per cent depression as derived from experiments in which the concentrations were varied from 10^{-6} M to 5×10^{-4} M. Incubation of the enzyme with the

TABLE I

Molar Concentration of Mercury Compounds Causing 50 Per Cent Depression of Succinic Dehydrogenase and Succinoxidase Systems

	Phenylmercuric nitrate	Phenylmercuric hydroxide	*p*-Chloromercuri-benzoic acid
Succinic dehydrogenase	3.2×10^{-5}	7.5×10^{-5}	4.0×10^{-5}
Succinoxidase......................	3.6×10^{-6}	5.0×10^{-6}	7.5×10^{-6}
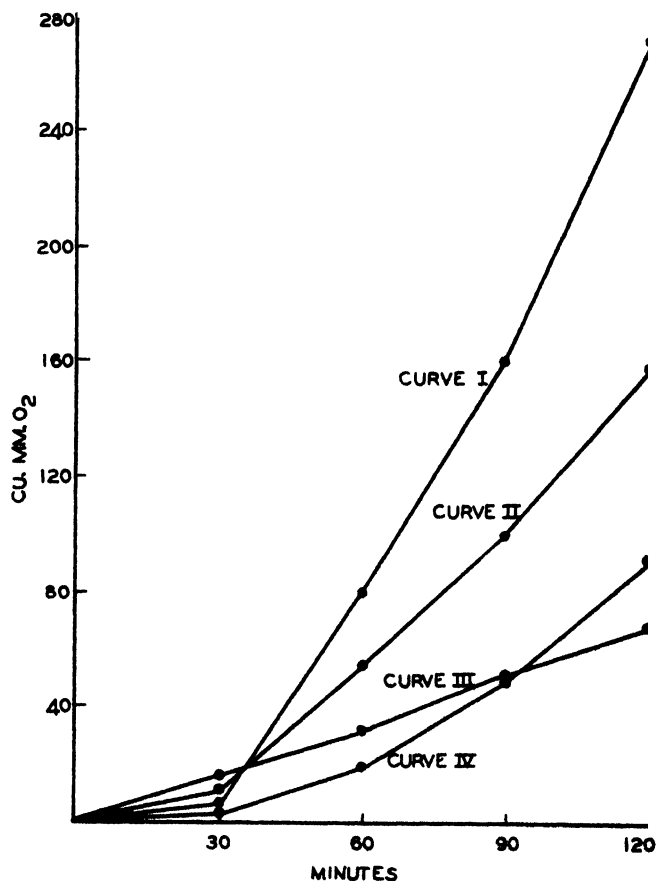
FIG. 1. Action of phenylmercuric nitrate, cysteine, and cystine on the succinoxidase system. Basic phenylmercuric nitrate, 2.5×10^{-5} M, present from the start; succinate added at 30 minutes. Curve I, control; Curve II, phenylmercuric nitrate present; Curve III, phenylmercuric nitrate and 10^{-4} M cysteine present; Curve IV, phenylmercuric nitrate and 5.0×10^{-5} M cystine present.

mercurial for 15 minutes increased the degree of inhibition by 30 to 50 per cent.

Incubation of the mercurial (10^{-5} M to 6×10^{-5} M) with 10^{-4} M or lower cysteine (higher concentrations decolorize methylene blue) before adding to the enzyme afforded protection, the degree of which varied with the relative concentrations of thiol and mercurial; molar concentrations of cysteine 5 to 10 times that of the mercurial were necessary for protection. Cysteine was unable to reverse the depression obtained after a 15 minute incubation of the enzyme with the mercurial. Cystine in concentrations of 10^{-4} M or lower (higher concentrations depressed) did not affect the inhibition caused by the mercurial.

Succinoxidase System—Table I shows that, under the conditions of the experiments, the aerobic system is considerably more sensitive to the mercurials than is the anaerobic, which is in accord with our earlier results (1) and those of Slater (2). In the aerobic system, unlike the anaerobic, cysteine afforded no protection against the mercurial inhibition even when present in 40 M excess. On the contrary, cysteine, in concentrations of 10^{-4} M to 10^{-5} M which in themselves did not affect the system, caused added depression when added simultaneously with the mercurial prior to the equilibration period, as illustrated in the typical experiment of Fig. 1, Curves I, II, and III. Incubation of the mercury compound with cysteine prior to addition to the system caused the same degree of inhibition as the mercurial alone. However, 10^{-4} M thioglycolic acid afforded about 35 per cent protection against phenylmercuric nitrate (2.5 to 6.0 $\times$ 10^{-6} M).

Cystine in non-depressing concentrations (5.0 $\times$ 10^{-5} M) added to the mercurial depression by about the same amount as an equivalent concentration of cysteine (Fig. 1).

DISCUSSION

The greater sensitivity of the aerobic system as compared with the anaerobic is attributed by Slater (2) not only to a combination of the mercurial inhibitor with the sulfhydryl group of the dehydrogenase but also to an effect on the protein particles of the enzyme preparation affecting the accessibility of the succinic dehydrogenase to cytochrome oxidase. This is in harmony with unpublished observations in our laboratory that the cytochrome oxidase system with ascorbic acid as a substrate is about as sensitive to the three mercurials as the anaerobic succinic dehydrogenase system. Mercurial concentrations of the order of 10^{-5} M, with only slight effect on the succinic dehydrogenase and cytochrome oxidase system, completely inhibited the succinoxidase system. Slater also reported *p*-chloromercuribenzoic acid to have some effect on cytochrome oxidase (2).

The ability of cysteine to protect against mercurial inhibition in the anaerobic but not in the aerobic system accords with the results of Hopkins and Morgan (8) and Barron and Singer (9) on the one hand, and of Potter and DuBois (10) and Ames and Elvehjem (11) on the other. Slater (3) suggests that in the presence of air the thiols inactivate the succinoxidase system by destroying a factor linking succinic dehydrogenase with cytochrome oxidase. Applying this suggestion to our results on the joint effects of thiols and mercurials in the aerobic system, one could postulate that the thiol compound would compete for reaction with the mercurial and the "linking factor" and that different thiol compounds would vary in their ability to protect the system or to inhibit it. Thus, the behaviors of cysteine and of thioglycolic acid differ. The additional inhibition caused by non-inhibitory concentrations of cysteine in the presence of the mercurials (unless incubated with them) may indicate that the mercurial has not only cut the effective concentration of the dehydrogenase but that by the action of mercurial and thiol sufficient linking factor has been destroyed or the accessibility of succinic dehydrogenase to cytochrome oxidase has been impaired so as to cause further inhibition.

That the oxidation of cysteine is involved in the inhibition of the aerobic system is suggested by the fact that 5.0×10^{-5} M cystine produced a similar added inhibitory effect to 10^{-4} M cysteine (Fig. 1). This accords with Slater's results (3) and with the findings of Barron et al. (4) that oxidized BAL inhibits succinoxidase and that the inhibitory action of dithiols on succinoxidase is correlated with the ease of oxidation of the dithiols.

SUMMARY

1. The succinoxidase system is more sensitive than the anaerobic succinic dehydrogenase system to the inhibitory action of basic phenylmercuric nitrate, phenylmercuric hydroxide, and p-chloromercuribenzoic acid.

2. In proper concentrations cysteine, thioglycolic acid, and cystine depress the succinoxidase system and cystine depresses the anaerobic system. In lower concentrations none of the thiol compounds depresses either system.

3. Cysteine, when present with the mercurials and the anaerobic enzyme system, protects against the depression caused by the mercurials. In non-depressing concentrations it does not protect the aerobic system but, on the contrary, causes added depression. The added depression of the aerobic system is avoided if the cysteine is incubated with the mercurial prior to their addition to the enzyme system. Thioglycolic acid affords partial protection of the aerobic system against the mercurials.

4. Cystine in concentrations which alone do not affect the anaerobic system has no effect on the depression caused by the mercurials. In the aerobic system concentrations of cystine which do not depress in them-

selves cause an added depression when present with the mercurials, thus resembling cysteine.

5. These findings are discussed in the light of the possible effects of the mercurials on the accessibility of the succinic dehydrogenase to the cytochrome oxidase and of the thiol compounds on a factor linking the succinic dehydrogenase and cytochrome oxidase systems.

BIBLIOGRAPHY

1. Cook, E. S., Kreke, C. W., McDevitt, M., and Bartlett, M. D., *J. Biol. Chem.*, **162**, 43 (1946).
2. Slater, E. C., *Biochem. J.*, **43**, p. xx (1948).
3. Slater, E. C., *Nature*, **161**, 405 (1948).
4. Barron, E. S. G., Miller, Z. B., and Meyer, J., *Biochem. J.*, **41**, 78 (1947).
5. Green, D. E., and Dixon, M., *Biochem. J.*, **28**, 237 (1934).
6. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, **149**, 217 (1943).
7. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **122**, 298 (1937).
8. Hopkins, F. G., and Morgan, E. J., *Biochem. J.*, **32**, 611 (1938).
9. Barron, E. S. G., and Singer, T. P., *J. Biol. Chem.*, **157**, 221 (1945).
10. Potter, V. R., and DuBois, K. P., *J. Gen. Physiol.*, **26**, 391 (1943).
11. Ames, S. R., and Elvehjem, C. A., *Arch. Biochem.*, **5**, 191 (1944).

PREPARATION OF THE OPTICALLY ACTIVE ISOMERS OF S-BENZYLHOMOCYSTEINE BY ENZYMATIC RESOLUTION*

By LESTER J. REED, A. R. KIDWAI,† AND VINCENT DU VIGNEAUD

(*From the Department of Biochemistry, Cornell University Medical College, New York City*)

S-Benzylhomocysteine, containing isotopic atoms, has proved to be a key intermediate in the synthesis of labeled methionine and related compounds (1). The need of the optically active isomers of these tagged compounds for various metabolic investigations has prompted us to work out a method for the resolution of S-benzylhomocysteine which would be particularly suitable for small quantities of S-benzylhomocysteine likely to be used in syntheses of this compound labeled with isotopes.

The success which attended the use of papain in the resolution of DL-methionine by Dekker and Fruton (2) encouraged us to explore the possibilities of this method for the preparation of the optically active isomers of S-benzylhomocysteine. When N-acetyl-S-benzyl-DL-homocysteine was incubated with aniline in the presence of cysteine-activated papain, N-acetyl-S-benzyl-L-homocysteine anilide crystallized in high yield. From the filtrate, there could be isolated N-acetyl-S-benzyl-D-homocysteine also in excellent yield. Recrystallization to satisfactory rotation gave a yield of 80 per cent for the L isomer and 89 per cent for the D isomer. Acid hydrolysis of these isomers has produced S-benzyl-L-homocysteine and S-benzyl-D-homocysteine of satisfactory purity in excellent yields. This enzymatic resolution method is equally effective with large or small amounts of S-benzyl-DL-homocysteine.

S-Benzyl-D-homocysteine has previously been prepared by du Vigneaud and Patterson (3) through the fractional crystallization of the brucine salts of the N-formyl derivatives of S-benzyl-DL-homocysteine. More recently, Dekker and Fruton (2) obtained S-benzyl-L-homocysteine by the acid hydrolysis of carbobenzoxy-L-methioninanilide from the enzymatic resolution of carbobenzoxy-DL-methionine.

EXPERIMENTAL

N-Acetyl-S-benzyl-DL-homocysteine—To a solution of 10 gm. (0.044 mole) of S-benzyl-DL-homocysteine in 13.2 cc. of water and 22.2 cc. of

2 N sodium hydroxide were added 106 cc. of 2 N sodium hydroxide and 10.6 cc. of acetic anhydride in ten portions during an interval of about 15 minutes. The reaction mixture was kept in an ice bath and stirred continuously during this time. After the mixture was stirred for an additional 20 minutes at room temperature, 47.7 cc. of 6 N sulfuric acid were added and the mixture was cooled. The crystals of *N*-acetyl-*S*-benzyl-DL-homocysteine were collected on a filter and washed with 33 cc. of 0.2 N hydrochloric acid and finally with water. The compound was crystallized from 15 per cent ethanol. A yield of 10.5 gm. (88 per cent of the theoretical amount) was obtained; m.p. 113.5–114.5°.[1]

$C_{13}H_{17}O_3NS$.   Calculated.   N 5.24, S 11.99
267.34   Found.   " 5.10, " 11.96

Enzymatic Synthesis of N-Acetyl-S-benzyl-L-homocysteine Anilide—To 9.9 gm. (0.037 mole) of *N*-acetyl-*S*-benzyl-DL-homocysteine were added 25 cc. of 1.08 N sodium hydroxide and 6.88 gm. (0.074 mole) of aniline. The undissolved material was brought into solution by adding 150 cc. of 0.2 M citrate buffer (pH 5.0) and warming the mixture. To this solution, cooled to room temperature, was added 0.56 gm. of L-cysteine hydrochloride dissolved in 20 cc. of buffer. A solution obtained by extracting 2.8 gm. of crude papain (dried papaya latex)[2] with 26 cc. of water was then introduced, followed by 114 cc. of buffer. Separation of the anilide began immediately. The flask was shaken vigorously to induce crystallization and then placed in a constant temperature room at 40° for 3 days. 6.23 gm. of crystalline product were obtained. A satisfactory purification could be effected by dissolving this material in hot acetone, filtering through a layer of norit, and evaporating the solvent. The solid remaining possessed a specific rotation of $[\alpha]_D^{22} = -24.0°$ (1 per cent in glacial acetic acid). After two recrystallizations from a 1:1 acetone-water mixture, 5.05 gm. of needle-like crystals melting at 152.5° were obtained; $[\alpha]_D^{21} = -24.9°$.

$C_{19}H_{22}O_2N_2S$.   Calculated.   N 8.18, S 9.36
342.45   Found.   " 8.19, " 9.08

N-Acetyl-S-benzyl-D-homocysteine—The filtrate from the enzymatic synthesis of *N*-acetyl-*S*-benzyl-L-homocysteine anilide was heated to boiling to coagulate the proteins, cooled, and then treated with norit. The yellow filtrate was concentrated *in vacuo* to approximately 100 cc. After acidification to Congo red with concentrated hydrochloric acid, *N*-acetyl-*S*-benzyl-D-homocysteine separated as an oil, which crystallized

[1] All melting points are corrected.
[2] Generously supplied by the Wallerstein Company, Inc., New York.

readily when it was cooled and scratched. The crystals were collected, washed with water, and dried over phosphorus pentoxide. A yield of 4.65 gm. was obtained. A solution of this material in 15 per cent ethanol was decolorized with norit, filtered, and cooled. 3.95 gm. of crystalline product were obtained, m.p. 131°. The specific rotation of a 1 per cent solution in glacial acetic acid was $[\alpha]_D^{21} = -14.6°$. A second recrystallization did not change the specific rotation.

$$C_{15}H_{17}O_2NS. \quad \text{Calculated.} \quad N\ 5.24,\ S\ 11.99$$
$$267.34 \qquad \text{Found.} \qquad \text{``}\ 4.94,\ \text{``}\ 11.54$$

S-Benzyl-L-homocysteine—A suspension of ·5.0 gm. (0.0146 mole) of *N*-acetyl-*S*-benzyl-L-homocysteine anilide, $[\alpha]_D^{21} = -24.9°$, in 350 cc. of 6 N hydrochloric acid was heated under a reflux for 6 hours. The resulting solution was concentrated *in vacuo* until solid material began to separate. This solid was redissolved by adding water and the solution was decolorized with norit, filtered, and made alkaline to Congo red and acid to litmus with ammonia. After being cooled for a few hours, the white crystals were collected and washed with ice water, followed by alcohol and ether. The yield of material possessing a specific rotation of $[\alpha]_D^{21} = +22.9°$ (1 per cent in 1 N hydrochloric acid) was 3.1 gm. (95 per cent of the theoretical amount). 3.0 gm. of this material were recrystallized from boiling water to give 2.6 gm. of crystalline product melting at 241–244°; $[\alpha]_D^{22} = +23.7°$. Du Vigneaud and Patterson (3) obtained a rotation of $[\alpha]_D^{22} = -25°$ (1 per cent in 1 N hydrochloric acid) for *S*-benzyl-D-homocysteine prepared by fractional crystallization of the brucine salts of the *N*-formyl derivatives of *S*-benzyl-DL-homocysteine, while the product obtained by Dekker and Fruton (2), by the acid hydrolysis of carbobenzoxy-L-methioninanilide from the enzymatic resolution of carbobenzoxy-DL-methionine, possessed a melting point of 243–244° and a rotation of $[\alpha]_D^{26} = +27.2°$ (1 per cent in 1 N hydrochloric acid).

$$C_{11}H_{15}O_2NS. \quad \text{Calculated.} \quad N\ 6.22,\ S\ 14.25$$
$$225.30 \qquad \text{Found.} \qquad \text{``}\ 6.04,\ \text{``}\ 14.31$$

S-Benzyl-D-homocysteine—2.67 gm. of *N*-acetyl-*S*-benzyl-D-homocysteine, $[\alpha]_D^{21} = -14.6°$, were hydrolyzed with 150 cc. of 6 N hydrochloric acid by the same procedure as that used for *N*-acetyl-*S*-benzyl-L-homocysteine anilide. From the hydrolysate, 2.17 gm. (96.4 per cent of the theoretical amount) of *S*-benzyl-D-homocysteine were obtained; $[\alpha]_D^{22} = -22.4°$ (1 per cent in 1 N hydrochloric acid). After one recrystallization from boiling water, 1.8 gm. of crystals melting at 240–243° were obtained; $[\alpha]_D^{22} = -23.2°$.

$$C_{11}H_{15}O_2NS. \quad \text{Calculated.} \quad N\ 6.22,\ S\ 14.25$$
$$225.30 \qquad \text{Found.} \qquad \text{``}\ 5.96,\ \text{``}\ 14.29$$

SUMMARY

The resolution of S-benzyl-DL-homocysteine by the action of papain on a mixture of N-acetyl-S-benzyl-DL-homocysteine and aniline has been presented. The anilide of the L isomer separates in crystalline form from the incubation mixture, and N-acetyl-S-benzyl-D-homocysteine may be isolated from the filtrate, both in excellent yield. Acid hydrolysis of these isomers produces S-benzyl-L-homocysteine and S-benzyl-D-homocysteine in excellent yields. The adaptability of this method to the resolution of S-benzylhomocysteine containing isotopes has been pointed out.

BIBLIOGRAPHY

1. Kilmer, G. W., and du Vigneaud, V., *J. Biol. Chem.*, **154**, 247 (1944). Tarver, H., and Schmidt, C. L. A., *J. Biol. Chem.*, **130**, 67 (1939); **146**, 69 (1942). Seligman, A. M., Rutenburg, A. M., and Banks, H., *J. Clin. Invest.*, **22**, 275 (1943). Patterson, W. I., and du Vigneaud, V., *J. Biol. Chem.*, **123**, 327 (1938).
2. Dekker, C. A., and Fruton, J. S., *J. Biol. Chem.*, **173**, 471 (1948).
3. du Vigneaud, V., and Patterson, W. I., *J. Biol. Chem.*, **109**, 97 (1935).

FURTHER STUDIES OF THE HARDEN-YOUNG EFFECT IN ALCOHOLIC FERMENTATION OF YEAST PREPARATIONS*

By OTTO MEYERHOF

WITH THE TECHNICAL ASSISTANCE OF JEAN R. WILSON

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

The Harden-Young effect of cell-free alcoholic fermentation is characterized by two kinetic phenomena: first, stoichiometric accumulation of hexose phosphates (hexose diphosphate and hexose monophosphate),[1] simultaneously with the fermentation of sugar, and secondly, a sudden decrease in the rate of fermentation after the free phosphate or the free sugar is exhausted. This effect is not observable in living yeast but only in preparations of dead yeast and is most pronounced in yeast extracts (press juice, Lebedev maceration juice, juice from yeast frozen in liquid air). It was shown some years ago (1) that the reason for the appearance of the effect is the inactivation of most of the ATPase or "apyrase" by the extracting procedure. In the presence of apyrase of potatoes, the effect is absent; *i.e.*, HDP ferments with the same speed as glucose. On the addition of apyrase after the break the fermentation curve of free sugar rises to the speed in the first period.

To elucidate these points further, it seemed worthwhile to try killing the yeast without damaging the ATPase[2] and to apply to such a preparation those substances which were recently found to be inhibitory to several types of ATPase (2, 3). The Harden-Young effect should be absent in such a yeast, but the application of these inhibitors might induce the

[1] Abbreviations used, HDP = hexose diphosphate; ATP = adenosine triphosphate; ADP = adenosine diphosphate; ATPase = adenosine triphosphatase, splitting one labile phosphate group; apyrase = adenylpyrophosphatase, splitting both labile phosphate groups; pyro P = the difference between 7 minute P and direct P; DPN = diphosphopyridine nucleotide or cozymase.

[2] Formerly (1), the ATP-splitting enzyme of yeast was called "apyrase." However, it was since found that with the purified enzyme only the first labile P group was split with great speed, the second group slowly. Because with further purifications probably only the first group would be split, this enzyme is now called ATPase according to the definition given in foot-note 1.

Harden-Young effect. Moreover, this study promised to shed light on the actual physical state of the ATPase in living yeast.

In the previous paper on this subject (1) it was assumed that Nilsson's so called "intakte Trockenhefe" (intact dry yeast) (4) was actually a yeast of the desired type and that the various procedures which he used to produce a break in the fermentation curve destroyed a large part of the ATPase. However, he misinterpreted his own results, and on account of his findings rejected the fermentation scheme worked out by the present writer (5).

Such an intact dry yeast was relatively easily obtained from American brewers' yeast. The application of the recently described ATPase inhibitors to this preparation gave the predicted results in a very satisfactory manner.

Methods and Procedures

For the experiments Schmidt's brewers' yeast[3] was used. The same type of dried yeast was also obtained from Ballantine's brewers' yeast. The yeast, after thorough washing with tap water, was suspended in a small volume of water; oxygen was bubbled through overnight in the cold room. This served to decrease the amount of storage material and to lower the autofermentation of the dry yeast.

After the yeast had been sucked as free of water as possible on a Büchner funnel, it was spread in a very thin layer on filter paper and warmed to 30–35°, so that it was completely dry after 4 hours. Such a yeast, which has undergone very little autolysis, has the desired properties of intact dry yeast, but, in distinction to the living cell, it is permeable to phosphate, arsenate, coenzymes, and phosphate esters.

In most experiments the dry yeast was resuspended in 12 volumes of water and used as such. In some cases it was subjected to sonic vibration by means of a Raytheon 9 kc. magnetostriction oscillator (type R-23-3, made of stainless steel and equipped with a water cooling system through which ice water flowed during the time of vibration). 2 gm. of dry yeast were suspended in a solution containing 12 cc. of water, 5 cc. of 0.1 M glutathione, and 1 cc. of 5.2 per cent $NaHCO_3$, and vibrated for 110 to 120 minutes.

Carbon dioxide was measured by Warburg's manometric technique at 29° in vessels of about 35 cc. capacity with two side arms. Sugar and the coenzymes were tipped in from one side. The gas space was filled with N_2 + 5 per cent CO_2.

Phosphate was determined by the Lohmann-Jendrássik modification (6)

---

of the Fiske-Subbarow procedure with an Evelyn photoelectric colorimeter and a 660 mμ filter. For the experiments on phosphorylation and ATPase inhibition, the yeast was shaken either in manometric vessels or in a similar device during the time of incubation. Otherwise the yeast clumped together and the measurement became inaccurate.

Results

The strong inhibitions of the *adsorbed* ATPase of the quickly dried yeast by saturation with octyl alcohol, toluene, and phenylurethane are shown in Table I (Samples 6 to 8) (inhibitions of 65 to 75 per cent). Smaller inhibitions (40 to 45 per cent) are caused by saturation with decyl alcohol and by 0.25 per cent digitonin. There is very little inhibition by saturation with tolylurea.

This contrasts with the effects on the *dissolved* ATPase of yeast with which octyl alcohol, toluene, and phenylurethane show no inhibition, and even some activation while in yeast frozen in liquid air; in yeast subjected to sonic vibration the ATPase is somewhat inhibited, although to a variable extent (Table I, Samples 3 and 4). It was recently shown (2, 3) that the same substances strongly inhibit the ATPase of malignant tumors, even in a dissolved or finely dispersed state.

Most of the pertinent features of fermentation of this type of dry yeast may be seen from Figs. 1 to 4. The fermentation rate of glucose (Fig. 1, Curve G) is practically constant until the amount of CO_2 formed (in excess of the blank value) is nearly equivalent to the sugar added: 8 mg. of sugar = 2000 c.mm. of CO_2 (arrow, Fig. 1). After a short transition period in which the remainder of the added sugar is exhausted (between 30 and 35 minutes), the course corresponds to the autofermentation shown by the blank (Curve B) in the absence of glucose. The fermentation rate of glucose is independent of the concentration of inorganic phosphate (below 0.1 M). In Fig. 1, Curve G, 500 γ of P of inorganic phosphate per 1.6 cc. were already present in the yeast (61.5 mg. dry weight). In a parallel experiment, in which the phosphate content was more than doubled by the addition of 620 γ of P of inorganic phosphate, the curve was identical. Only when 3 mg. of P of inorganic phosphate or more were added was the rate somewhat diminished. However, the situation was quite different after inhibition of the ATPase. With added octyl alcohol, the curve of fermentation depends on the amount of phosphate. Curve O shows fermentation with added octyl alcohol in the presence of the 500 γ of P of inorganic phosphate contained in the yeast. Curves O + P and O + 2P correspond to the further addition of 770 and 1440 γ of P of inorganic phosphate respectively. The addition of 770 γ of P allows the formation of 2.5×10^{-2} equivalents of ester phosphate, and should correspond to 560 c.mm. of extra CO_2

according to the Harden-Young equation. Actually 480 c.mm. of CO_2 are formed (difference between the ordinates for Curve O and Curve O + P

TABLE I

Inhibition of Yeast ATPase

ATP added   145 γ of pyro P = 4.7 μM.

Sample No.	Treatment of yeast	Temperature	Dry weight of yeast	Time of incubation	Inhibitor added	Concentration	Pyro P split	Per cent change
		°C.	mg.	min.		per cent	γ	
1	Purified ATPase	30	10	15			70	
				15	Octyl alcohol	Saturated	68	0
				15	Toluene	"	66.5	0
2	" "	38	25	10			105	
				10	Phenylurethane	Saturated	120	+15
3	" "	30	18	6			21.0	
			18	15			49	
			18	6	Octyl alcohol	Saturated	38	+80
			18	15	" "	"	68	+40
4	Supernatant of sonic vibration	30	14	5			44.5	
				5	Octyl alcohol	Saturated	32	−28
				5	Toluene	"	25	−44
				5	Decyl alcohol	"	30	−32
5	Liquid air-frozen particles	30	20	15			38	
				15	Octyl alcohol	Saturated	26.6	−30
				15	Toluene	"	31.8	−16
				15	Digitonin	0.15	29.5	−22
6	Quickly dried yeast	26	10	6			47.7	
				6	Octyl alcohol	Saturated	15.8	−67
				6	Phenylurethane	0.17	19.6	−59
				6	"	Saturated	16.5	−65
				6	Phenylurea	0.65	26	−45
				6	Tolylurea	Saturated	37.3	−22
	" " "	30	10	6			43.4	
			10	12			60.4	
7			10	6	Decyl alcohol		25.9	−40
			10	12	" "		34.0	−44
			10	6	Digitonin	0.25	23.6	−46
			10	12	"	0.25	35.5	−41
8	" " "	30	10	6			43.9	
			10	12			58.8	
			10	6	Octyl alcohol	Saturated	11.2	−74
			10	6	Toluene	"	14.0	−69
			10	12	"		16.7	−72

in the second, slow period). The same proportion holds for smaller amounts of inorganic phosphate. If, however, the added amount is fur-

ther increased (as in Curve O + 2P), then very little more CO_2 is formed in the rapid period. This again is in accordance with the Harden-Young effect, because now one-half of the added free sugar has been fermented, equal to 1000 c.mm. of CO_2, and the second half is esterified to hexose phosphate, so that no free sugar remains available.
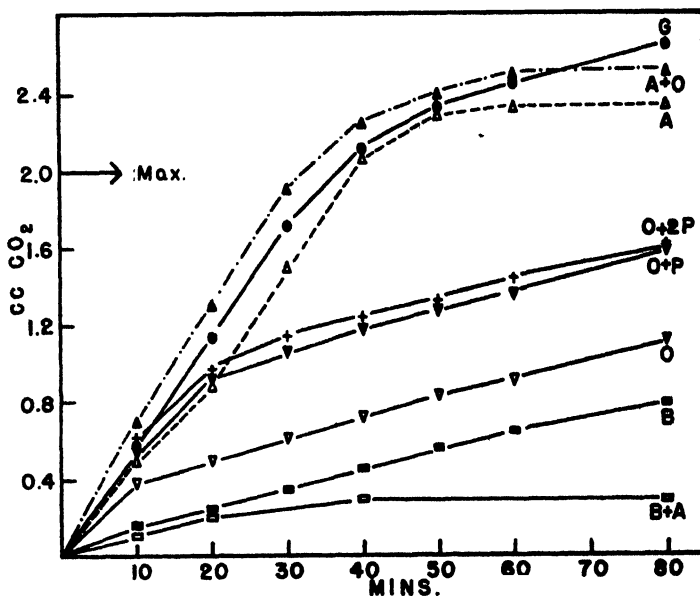
Fig. 1. Fermentation of glucose by quickly dried yeast. 8 mg. of glucose added corresponding to 2.0 cc. of CO_2. Gas space, N_2 + 5 per cent CO_2. All samples contain 0.75 cc. of yeast suspension (61.5 mg., dry weight) made up to 1.0 cc. by various additions in the main compartment. At zero time 0.6 cc. of a solution is tipped in from the side arm with the following ingredients: 8 mg. of glucose, HDP containing 30 γ of P, 400 γ of DPN, ATP with 65 γ of pyro P, 1.5 mg. of acetaldehyde, 6 mg. of Mg as $MgSO_4$. In Curves B and B + A the same solution without glucose. Solid curves: Curves G, B, and B + A, no further additions; Curve O, saturated octyl alcohol; Curve O + P, saturated octyl alcohol + 0.25 cc. of 0.1 M KH_2PO_4; Curve O + 2P, saturated octyl alcohol + 0.25 cc. of 0.2 M KH_2PO_4. Dotted curves: Curve A, 0.25 cc. of 0.04 M arsenate; Curve A + O, 0.25 cc. of 0.04 M arsenate with saturated octyl alcohol. The arrow at 2 cc. shows the maximum amount of CO_2 which can form from the added glucose.

By the addition of arsenate it can be strikingly demonstrated that octyl alcohol and toluene do not inhibit other enzymes besides the ATPase. Fermentation in the presence of arsenate differs from ordinary fermentation only in the formation of 1-arseno-3-phosphoglyceric acid instead of 1,3-diphosphoglyceric acid (7) and by the spontaneous dephosphorylation

of phosphopyruvate by way of ADP and the "coupling reaction" (8). All the enzymatic steps are the same in both cases except that the transphosphorylase from 1,3-phosphoglyceric acid to ADP and the ATPase are not needed. In the presence of arsenate, the fermentation of our dry yeast is not inhibited by octyl alcohol, toluene, and phenylurethane. Actually it is somewhat increased in the rapid period (see Fig. 1, Curves A and A + O). (The blank rate with arsenate (Curve B + A) soon slows consider-
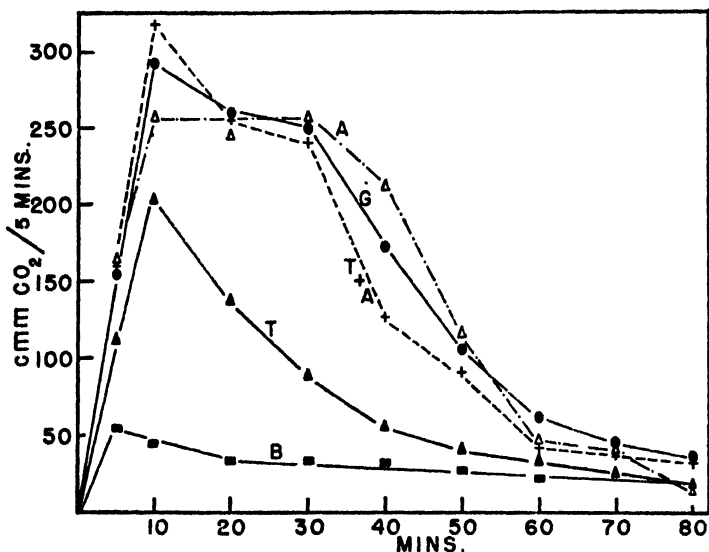
FIG. 2. Rate of fermentation of quickly dried yeast. Set up similar to the experiment in Fig. 1. All samples contain 0.5 cc. of yeast suspension (41 mg., dry weight), 0.4 cc. of boiled yeast juice and 0.3 cc. of additions in main compartment. At zero time 0.7 cc. of a solution is tipped in containing the same amounts as in the experiment in Fig. 1, but only 200 γ of DPN. Solid curves: Curve G, no further addition; Curve T, saturated toluene; Curve B, blank without glucose. Dotted curves: with 0.15 cc. of 0.04 M arsenate per sample, Curve A, arsenate alone; Curve T + A, arsenate with saturated toluene.

ably and, accordingly, the curves with sugar also become horizontal after 50 to 60 minutes.)

A similar result is obtained with all ATPase inhibitors. This can be seen in Figs. 2 and 3 where the speed (c.mm. of CO_2 per 5 minutes) is reproduced for the various cases cited. In Fig. 2 with toluene (Curve T) and Fig. 3 with phenylurethane (Curve P) the initial speed of the arsenate samples is actually increased (Curves T + A and P + A, as in Fig. 1, Curve A + O); the rates, therefore, fall off sooner because of the more rapid exhaustion of the added sugar.

The analysis of the phosphate turnover simultaneously with the measurement of CO_2 confirms the conclusion that it is not the transphosphorylase but the ATPase that is inhibited, and that this inhibition causes the break in the fermentation curves. In the absence of the inhibitors the inorganic phosphate does not change, or it increases slightly as a result of decomposition of stored phosphate esters, while with the inhibitors a strong esterification occurs. The ester consists of from one-half to two-thirds of HDP, the remainder being hexose monophosphate. Such an experiment is shown in Fig. 4 and the analysis of HDP by means of zymohexase is given
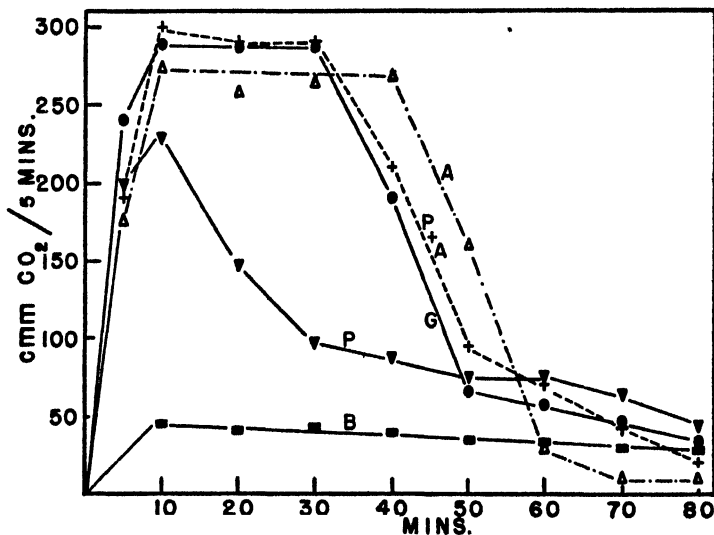
FIG 3. Rate of fermentation of quickly dried yeast. All samples contain 0.75 cc. of yeast suspension (62 mg., dry weight), no boiled juice, and the same additions as in the experiment of Fig. 1. Solid curves: Curve B, blank without glucose; Curve G, only glucose; Curve P, glucose with saturated phenylurethane. Dotted curves with 0.15 cc. of 0.04 M arsenate: Curve A, arsenate alone; Curve P + A, arsenate with saturated phenylurethane.

in Table II. For the experiments reproduced in Fig. 4 several parallel runs were made, and every 10 minutes a vessel was removed for phosphate determination; the disappearance of inorganic phosphate is drawn in dotted lines (additional experiments are given in Table II).

Some qualifications may be mentioned: our quickly dried yeast does not come into equilibrium with the phosphate compounds outside the cells as readily as do yeast preparations which are made comp'etely permeable by autolysis or by freezing in liquid air (1). $Q_{CO_2}^{N_2}$ of fermentation for the rapid period is about 70 at 29°, depending on the concentration of coen-

zymes, but Q_P for ATPase[4] is only about 30, presumably because ATP does not enter the cells quite freely. Moreover, $Q_{CO_2}^{N_2}$ for HDP is only
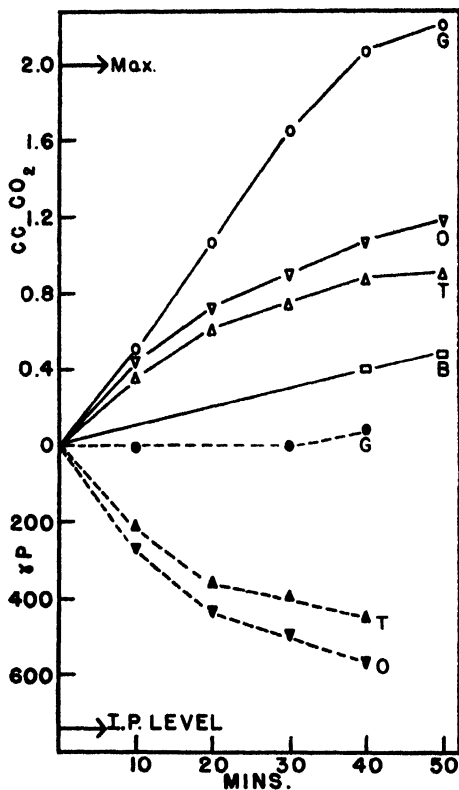
Fig. 4. Fermentation of glucose and phosphate turnover. 8 mg. of sugar added corresponding to 2.0 cc. of CO_2 (arrow). The phosphate turnover, measured by the disappearance of inorganic phosphate (γ of P per sample) is drawn downward (dotted curves). Composition similar to experiment in Fig. 2. All samples contain 0.5 cc. of yeast suspension (41 mg., dry weight), 0.4 cc. of boiled juice, 0.5 cc. of distilled H_2O in the main compartment. At zero time 0.5 cc. of a solution containing the following ingredients is tipped in: 8 mg. of glucose, HDP with 30 γ of P, 200 γ of DPN, ATP with 64 γ of pyro P, 1.5 mg. of acetaldehyde, 6 mg. of Mg as $MgSO_4$. Solid curves: CO_2. Dotted curves: inorganic phosphate. Curves G, no further addition; Curves T, toluene; Curves O, octyl alcohol; Curves B, blank without glucose. The arrow at the bottom (I. P. level) indicates the total amount of inorganic phosphate available for esterification.

about 25 and is very little increased by arsenate. In yeast preparations which are freely permeable, HDP ferments as quickly as free sugar in the

---

[4] Q_P = c.mm. of H_3PO_4 split off from ATP at 30° in 1 hour per mg. of dry weight of yeast (22.4 c.mm. = 31 γ of P).

presence of arsenate; or with an excess of ATPase even in the absence of arsenate (1).

Probably for the same reason, the amount of hexose phosphates accumulating in experiments of the type shown in Fig. 4 is often not equivalent to the decrease in the fermentation rate in the presence of the ATPase inhibitors, but may be only about one-half of this value.

TABLE II

Esterification of Phosphate and Formation of Hexose Diphosphate during Fermentation (29°)

The numbers indicate micromoles of CO_2 or phosphate.

Sample No	Dry weight of yeast	Inhibitor added (saturated)	Time	Inorganic phosphate present	Phosphate esterified	HDP formed	CO₂ formed minus blank
	mg.		*min.*				
1*	42		0	24.6			
			40	27.8		1.2	74
			90	36.2		0	83
		Octyl alcohol	40	8.0	16.6	11.8	29
		" "	90	18.4	7.4	6.0	31
		Toluene	40	9.5	14.5	7.7	16
		"	90	23.6	1.6	1.2	
2	42		0	9.6			
			120	11.3		0	74
		Decyl alcohol	120	2.8	6.8	6.1	34
3	62		0	12.7			
			10				26
			40	18.4			75
		Phenylurethane	10	3.3	9.4		15.8
		"	20	1.6	11.1		23.8
		"	40	1.3	11.4		33.0
		Octyl alcohol	40	1.0	11.7		15.0
4	42		0	18.5			
			10	18.5			19.0
			30	18.7			67.5
		Digitonin†	10	12.8	5.7		15.5
		"	30	8.5	10.0		35.5

* Plus boiled yeast juice.
† 0.3 per cent.

If the quickly dried yeast is subjected to sonic vibration and the cell débris is centrifuged out, the fermentation of the "sonic solution" shows nearly the same features as the "sonic solution" prepared from living yeast. The fermentation rate of free sugar, which is initially high, falls quickly, while the inorganic phosphate present is completely esterified in 10 minutes. In the presence of arsenate the high speed is maintained much longer. Still, such a "sonic solution" contains more ATPase than an or-

dinary preparation of yeast extract. On account of this, octyl alcohol and phenylurethane decrease this speed in the second period to a considerable degree. While the phosphate ester in the absence of narcotics is rapidly split again during the next 20 to 30 minutes, with octyl alcohol the total amount of ester remains nearly constant, with a very low level of inorganic phosphate, as long as sugar is available. This shows that the "sonic solution," although it has lost part of the ATPase, still contains appreciable amounts of the enzyme (see Table III). While the enzyme in this condition shows some inhibition with octyl alcohol and phenylurethane, this inhibition disappears completely on further purification. The exact reverse effect is obtained with sodium azide. The purified ATPase is inhibited 65 per cent by 10^{-4} M azide (1). However, in the quickly dried

TABLE III

Esterification of Phosphate and CO_2 Production in Supernatant of Sonic-Vibrated Dry Yeast (Supernatant from 80 Mg. of Dry Powder)

Temperature 29°; all values in micromoles; 8 mg. of sugar added equivalent to 89 μM of CO_2.

Additions	Time	Inorganic phosphate present	Phosphate esterified	CO₂ production minus blank
	min.			
Glucose	0	17.7		
	10	1 53	−16.2	32.6
	30	20		60.5
	50	23.8		71.0
" + octyl alcohol	10	0.62	−17.1	22.4
	30	0.67	−17.0	28.0
	50	0.87	−16.8	35.0
Blank (no glucose)	0	23.2		(11.3)*

* CO_2 formed without sugar.

yeast even 2×10^{-2} M does not inhibit, possibly because of the influence of foreign protein (see a similar case, Spiegelman et al. (9)).

DISCUSSION

After the preceding publication (1) no reasonable doubt could exist that the Harden-Young effect was caused by a lack of ATPase. The present paper proves conclusively that the ATPase is partly destroyed by the procedures which are applied to bring the fermenting system into solution, especially by the autolysis of the yeast and the breaking up of the cell structures. The substances used for quick autolysis like toluene and octyl alcohol are themselves very strong inhibitors of the adsorbed ATPase, as

has been shown in the foregoing. Nearly all the features described by Nilsson for his own intact dry yeast are also exhibited by the preparation used here. The breaks in the fermentation curves evoked by different influences can always be explained by the removal, destruction, or inhibition of the ATPase.

The difference in the susceptibility of the same enzyme to high members of the narcotic series, according to whether it is in solution or is adsorbed, is a striking confirmation of the old theory of Warburg (10) that, compared with the enzyme extracts, the greater sensitivity of respiration and fermentation of living cells to these narcotics is caused by adsorption of the enzymes at the interphases of the living cell. As far as I know, this theory has never been put to a test for an individual enzyme in a dissolved and adsorbed state. Our experiments show that the theory is exactly true for the yeast ATPase. However, it is probable that not all enzymes behave in the same way. On the one hand, the tumor ATPase in solution or in a fine dispersion is inhibited to the same degree by these narcotics; on the other hand, the rule apparently does not apply to the other glycolytic enzymes which are not inhibited in our dry yeast.

SUMMARY

The fermentation of glucose in quickly dried brewers' yeast proceeds without a break in the rate of fermentation until as much CO_2 is produced (in excess of the blank) as corresponds to the complete fermentation of the sugar. At the same time no phosphate esters accumulate; on the contrary, the inorganic phosphate increases somewhat by the breakdown of the stored organic esters.

If the ATPase inhibitors are applied to this yeast, the Harden-Young effect appears; the break in the fermentation curve occurs if either free phosphate or the free sugar is exhausted. At the same time, hexose esters, mostly hexose diphosphate, accumulate. The adsorbed ATPase in the quickly dried yeast is inhibited by higher members of the narcotic series: octyl alcohol, phenylurethane, and toluene. If the adsorbed yeast ATPase is brought into solution and partially purified, inhibition no longer occurs.

In the presence of arsenate no break in the fermentation rate of the dry yeast is caused by these ATPase inhibitors, because here the ATPase is not needed for dephosphorylation.

After sonic vibration of the quickly dried yeast the supernatant solution shows the Harden-Young effect, although it is still not quite complete. Even in this case the narcotics cause a sharper break in the curve, and the accumulation of phosphate esters becomes more pronounced. These facts prove that a removal, destruction, or inhibition of ATPase is responsible for the Harden-Young effect in yeast preparations.

BIBLIOGRAPHY

1. Meyerhof, O., *J. Biol. Chem.*, **157**, 105 (1945).
2. Meyerhof, O., and Wilson, J. R., *Arch. Biochem.*, **21**, 1, 22 (1949).
3. Meyerhof, O., and Wilson, J. R., *Arch. Biochem.*, in press.
4. Nilsson, R., and Alm, F., *Biochem. Z.*, **286**, 254, 373 (1936); **304**, 285 (1940); **308**, 255 (1941).
5. Nilsson, R., *Naturwissenschaften*, **31**, 23 (1943).
6. Lohmann, K., and Jendrássik, L., *Biochem. Z.*, **178**, 419 (1927).
7. Warburg, O., and Christian, W., *Biochem. Z.*, **301**, 22 (1939); **303**, 40 (1939).
8. Meyerhof, O., and Junowicz-Kocholaty, R., *J. Biol. Chem.*, **145**, 443 (1942).
9. Spiegelman, S., Kamen, M. D., and Sussman, M., *Arch. Biochem.*, **18**, 409 (1948).
10. Warburg, O., *Ergeb. Physiol.*, **14**, 253 (1914).

GLYCOLYSIS IN LIVER HOMOGENATES*

By PAUL A. STOESZ† AND G. A. LePAGE

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison)

Recently, studies of anaerobic glycolysis in our laboratory led to the development of a medium for maintaining phosphate bond energy in homogenates of tumor (1). This made possible ·the study of synthetic reactions requiring such an energy supply. Subsequently it became necessary to have a medium in which homogenates of rat liver would maintain phosphate bond energy under anaerobic conditions. While a number of investigations of anaerobic glycolysis have been made with rat liver slices, the most recent by Warren and Ebaugh (2), very little information is available concerning glycolysis in homogenates of liver. Reiner provided some data concerning rat liver homogenates (3) and found several differences in behavior compared to brain homogenates. He was unable to obtain linearity with tissue concentration and reported irregularities in rate.

In this study the medium of LePage (1) for tumor glycolysis was first used, and constituents were varied singly to determine the optimum for each. Then variations in each were studied in the presence of the optima of all other factors. The carbon dioxide liberation from a bicarbonate buffer was followed as a general indication of glycolysis rate, but the criteria used in evaluation of media were lactic acid production and net phosphorus esterification as measured by chemical methods.

EXPERIMENTAL

Albino rats of both sexes obtained from the Holtzman Rat Company, weighing 190 to 270 gm., were used throughout these experiments. Homogenates were made with Potter-Elvehjem homogenizers in 9 volumes of cold isotonic potassium chloride with precautions which have been discussed previously (1, 4). All constituents of the medium were pipetted into Warburg flasks and the flasks were immersed in ice before homogenate was added. The gas phase was 95 per cent nitrogen-5 per cent carbon dioxide. Gassing was carried out as described previously (1). The maximum time from removal of a given flask from ice until incuba-

tion was begun in a bath at 37.6° was 5.5 minutes. Flasks were equilibrated 5 minutes before readings were begun.

Reaction mixtures were inactivated at the indicated times by addition of 0.25 ml. of 65 per cent trichloroacetic acid from the side arm of each flask. The flask contents were in each case transferred to a small test-tube and the precipitated protein centrifuged. Lactic acid and inorganic phosphorus were determined on the supernatant fluid by methods descriLed earlier (5).

Preparations of diphosphopyridine nucleotide (DPN) were 84 to 88 per cent pure; those of adenosine triphosphate (ATP) were 98 per cent pure (6). Hexose diphosphate (HDP) was obtained as the dibarium salt from the Schwarz Laboratories and further purified by the method of Neuberg *et al.* (7). Pyruvic acid was distilled at 2 to 4 mm. of Hg pressure and immediately diluted and stored at 0° as a 1 M solution. All the above preparations were used as potassium salts.

TABLE I

Effects of Varied Tonicity in 40 Minute Incubation with 20 Mg. of Liver per Flask

Index of tonicity	Lactic acid produced	Net P esterified
	μM	μM
0.86*	11.22	−1.90
1.00	11.09	−1.42
1.30	10.85	−0.61

* Selected for final medium.

A large number of preliminary experiments were conducted to determine the approximate optima of constituents of the medium. Then each constituent was varied with all others optimum. Only the latter results are presented here. Each figure on the charts or tables in general represents an average of single or duplicate flasks, usually duplicate, in each of two experiments. Variations between experiments were relatively small with the exception of one group of rats encountered during preliminary work, which gave uniform glycolytic rates at lower levels (20 to 40 per cent lower).

Tonicity of Medium—If the value of 1.0 is assigned to an isotonic KCl solution, the tonicity of the medium used by LePage (1) was 0.65. By adding different amounts of KCl to the medium, various higher tonicity levels were produced. The results of lactic acid production and net phosphorus esterification in Table I were obtained. The tonicity of the medium, optimum in all other respects, for liver homogenates was calculated to be 0.86. It appeared unnecessary to modify this.

Initial pH of Medium—By varying the concentration of KHCO₃, different pH values can be obtained. Fig. 1 shows the effect produced by altering the initial pH through a range of 7.3 to 8.13. The optimum selected was pH 7.8. The final pH after a 40 minute incubation is approximately 0.2 pH unit lower than the initial pH.

Substrate—Warren and Ebaugh (2) found that anaerobic glycolysis of liver slices was unaffected by the presence or absence of glucose. This has been confirmed for our liver homogenates and consequently glucose was deleted from the medium. It was necessary to use HDP as the main substrate. Fig. 2 indicates the effects of various HDP concentrations during a 40 minute incubation. Glycolysis was found to proceed with
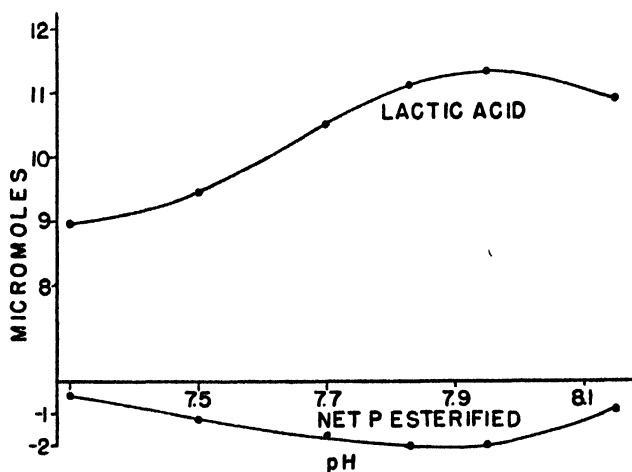
Fig. 1. Effects of varied initial pH on lactic acid produced and net phosphorus esterified per flask during a 40 minute incubation with 20 mg. of rat liver.

equal rapidity with or without added fluoride in the presence of pyruvate. Since the use of fluoride has the advantage of preserving phosphate bond energy, thereby facilitating the evaluation of phosphorus balances, we decided to block the glycolytic reactions at the phosphoglyceric acid stage and use the triose phosphate dehydrogenase reaction as the source of energy. Since measurements of triose phosphate[1] in the reaction mixtures indicate that it accumulates slightly, the zymohexase must be in excess in liver and it seems likely that we are actually measuring triose phosphate dehydrogenase as the limiting reaction.

Pyruvate—Since fluoride is added to this sytem to block conversion of phosphoglyceric acid to pyruvic acid, the latter had to be added as hydro-

[1] Unpublished data.

gen acceptor.   Table II shows the effect of varying amounts of pyruvate
on glycolysis during a 40 minute incubation.
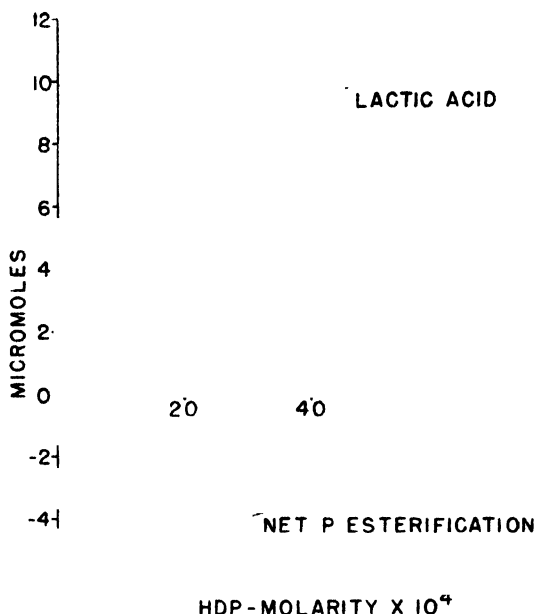
FIG. 2. Effects of varied HDP concentration on lactic acid produced and net
phosphorus esterified per flask in a 40 minute incubation with 20 mg. of rat liver.

TABLE II

*Effects of Variations in Pyruvate on Glycolysis with 20 Mg. of Rat Liver in 40 Minute
Incubation*

Pyruvate concentration	Lactic acid produced	Net P esterified
M	μM	
0	0.42	−2.22
0.0025	5.79	−1.68
0.005	10.42	−0.61
0.0075*	10.47	−0.74
0.01	10.20	−0.71
0.015	9.54	−0.74

' Selected for final medium.

Magnesium and Fluoride Ions—The fluoride concentration chosen here
results in near saturation with MgF_2 (8); however, no precipitation of
MgF_2 was noted at any time.

Tables III and IV show the effect of varying amounts of Mg^{++} and
F^- ions on glycolysis during a 40 minute incubation.

It may be noted that higher concentrations of fluoride increased net P esterification. In the absence of fluoride the loss of esterified phosphate corresponds to the amount which would be obtained by complete hydrolysis of the added ATP to adenylic acid.

ATP—Data in Fig. 3 show the effects of varied ATP concentrations. The optimum selected was 3 times that selected for tumor glycolysis (1). This probably results from the inability of the liver homogenates to utilize the high energy phosphate to phosphorylate glucose and thereby

TABLE III

Effects of Variations in Added Magnesium Chloride on Glycolysis by 20 Mg. of Rat Liver in 40 Minute Incubation

MgCl₂ concentration	Lactic acid produced	Net P esterified
	μM	*μM*
0	7.93	−1.35
0.0005	9.87	−2.55
0.001*	10.61	−1.94
0.002	11.72	−1.81
0.004	11.17	−1.26
0.008	10.52	−2.48

* Selected for final medium.

TABLE IV

Effects of Variations in Potassium Fluoride on Glycolysis by 20 Mg. of Rat Liver in 40 Minute Incubation

KF concentration	Lactic acid produced	Net P esterified
M	*μM*	*μM*
0	9.30	−4.06
0.005	9.84	−2.36
0.01*	9.80	−1.55
0.02	9.42	−0.39
0.03	8.81	+0.77
0.04	8.46	+0.99

* Selected for final medium.

make phosphate acceptor available. The liver homogenate preparation is dependent upon ATPase to make phosphate acceptor available. Adenylic acid gave results comparable to ATP, but the net phosphorus esterification was increased.

DPN—Data obtained by varying DPN concentration during a 40 minute incubation appear in Fig. 4. The concentration chosen for optimum lactic acid and net P esterification values was 0.0003 M.

Nicotinamide—In order to prevent depletion of the DPN, as pointed
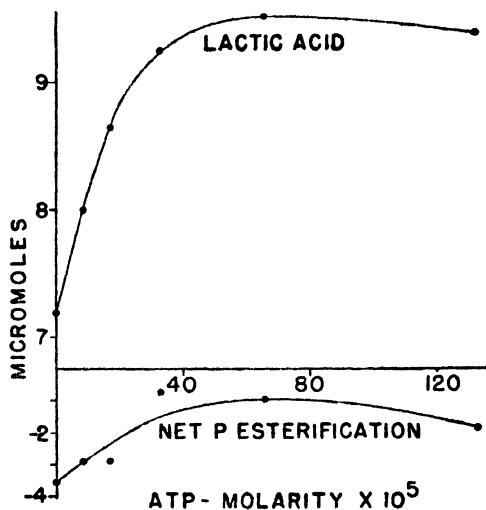
FIG. 3. Effects of varied ATP on lactic acid production and net phosphorus es‑
terification per flask in 20 mg. of rat liver during a 40 minute incubation.
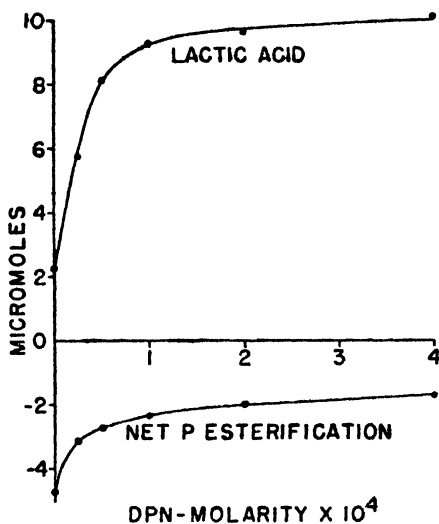
FIG. 4. Effects of varied DPN concentration on lactic acid production and net
phosphorus esterification per flask in a 40 minute incubation with 20 mg. of rat liver.

out by Novikoff *et al.* (4), the system was tested with varying amounts
of nicotinamide. It may be concluded from Table V that some protection
of DPN was obtained with nicotinamide and that 0.04 M nicotinamide

provides maximum protection.   No added protection was obtained with α-tocopherol phosphate.

Homogenate—In preliminary studies, 30 mg., wet weight, of liver tissue were used; however, it became apparent that 20 mg. gave more suitable

TABLE V

Effects of Nicotinamide Additions on Glycolysis by 20 Mg. of Rat Liver in 40 Minute Incubation

Nicotinamide concentration	Lactic acid produced	Net P esterified
M	*μM*	*μM*
0	7.75	−1.55
0.02	8.39	−1.32
0.04*	8.85	−0.97

' Selected for final medium.
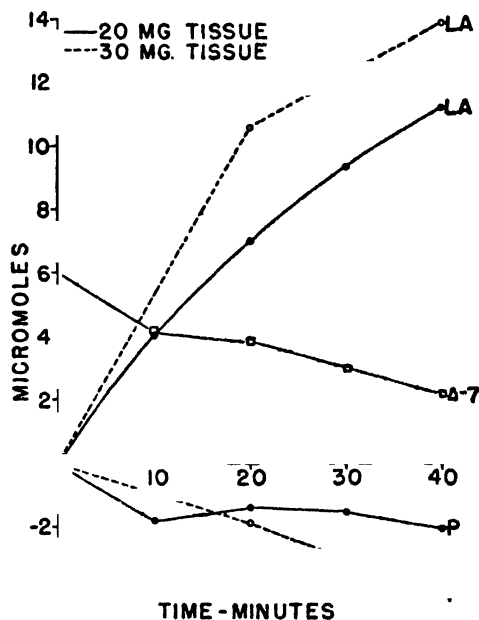
FIG. 5. Effects of varied incubation time and tissue concentration on lactic acid production, net phosphorus esterification, and ATP-pyrophosphate phosphorus.

rates.   Fig. 5 illustrates the linearity obtained when 20 and 30 mg. of tissue were incubated in the optimum medium for 20 minutes.   When the incubation was extended to 40 minutes, linearity was not perfect owing to the fact that HDP became limiting.

The composition of the medium selected was as follows: potassium phosphate 0.0016 M, potassium bicarbonate 0.050 M, nicotinamide 0.040 M, pyruvic acid 0.0075 M, adenosine triphosphate 0.001 M, hexose diphosphate 0.006 M, diphosphopyridine nucleotide 0.0003 M, magnesium chloride 0.001 M, potassium fluoride 0.010 M.

Rate of Reaction—During a 40 minute run, duplicate flasks were inactivated every 10 minutes. Measurements were then made of lactic acid production, net P esterification, and ATP pyrophosphate phosphorus. The latter was measured by the method described by Potter *et al.* (9). These results appear in Fig. 5. ATP phosphorus was observed to drop to two-thirds of the original value during the first 20 minutes and to one-third by 40 minutes.

DISCUSSION

Reiner (3) attempted to find a suitable medium for the anaerobic glycolysis of homogenized rat liver, but was only partially successful. His value[2] of $Q_L^{N_2}$ was approximately 23, whereas calculations from data on this system reveal $Q_L^{N_2}$ to be approximately 78. Reiner succeeded in getting slightly higher values when nicotinamide and HDP were increased. He was using a system which proceeded through to lactic acid, without fluoride to block the reactions at the phosphoglyceric acid stage. However, in our medium glycolysis was equally rapid with and without fluoride (see Table IV). It therefore seems unlikely that Reiner's lower rates were due to a limitation imposed by the enzymes which convert phosphoglyceric acid to pyruvic acid.

It is evident that glucose is not phosphorylated during glycolysis by livers obtained from intact rats. When hexose diphosphate is used as substrate, the energy system can be maintained.

Reiner (3) reported that he obtained more rapid glycolysis in brain than in liver. However, the medium developed here permits liver glycolysis to proceed at a rate which exceeds any rates reported for rat brain homogenates.

SUMMARY

A medium for anaerobic glycolysis of rat liver has been developed in which hexose diphosphate is the substrate. The $Q_L^{N_2}$ obtained was approximately 78. Phosphate bond energy has been shown to be maintained at satisfactory levels.

[2] Mg. of lactic acid produced anaerobically per hour per mg. dry weight of liver. Liver dry weights are approximately 30 per cent of the wet weight.

BIBLIOGRAPHY

1. LePage, G. A., *J. Biol. Chem.*, **176,** 1009 (1948).
2. Warren, C. O., and Ebaugh, F. G., *Am. J. Physiol.*, **147,** 509 (1946).
3. Reiner, J. M., *Arch. Biochem.*, **12,** 327 (1947).
4. Novikoff, A. B., Potter, V. R., and LePage, G. A., *Cancer Res.*, **8,** 203 (1948).
5. Umbreit, W. W., Burris, R. H., and Stauffer, J. W., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1945).
6. LePage, G. A., in Carter, II. E., Biochemical preparations, New York (1949).
7. Neuberg, C., Lustig, II., and Rothenberg, M. A., *Arch. Biochem.*, **3,** 33 (1943–44).
8. Potter, V. R., *J. Biol. Chem.*, **169,** 17 (1947).
9. Potter, V. R., LePage, G. A., and Klug, H. I. , *J. Biol. Chem.*, **175,** 619 (1948).

DIPHTHERIA TOXIN

V. A COMPARISON BETWEEN THE DIPHTHERIAL SUCCINOXIDASE SYSTEM AND THAT OF BEEF HEART MUSCLE[*]

By ALWIN M. PAPPENHEIMER, Jr., AND EDELMIRA D. HENDEE

(From the Department of Microbiology, New York University College of Medicine, New York)

The major iron-containing respiratory pigment of *Corynebacterium diphtheriae* is spectroscopically related to cytochrome *b* (1-3). In a previous communication (2) we presented evidence which suggests that diphtheria toxin may be closely related to the protein moiety of diphtherial cytochrome *b*. The tentative hypothesis was advanced that diphtheria toxin may act by interfering with the normal function of cytochrome *b* in the tissues of the susceptible host, possibly by blocking its synthesis.

Before attempting to verify this hypothesis, it has seemed necessary to investigate further the rôle of cytochrome *b* both in mammalian tissue respiration and in the bacterial cell. There seems to be little doubt that cytochrome *b* is concerned in the oxidation of succinate by heart muscle succinoxidase preparations in which it may serve as a link between succindehydrogenase and the cytochrome *c*-cytochrome oxidase system (Keilin and Hartree (4); Ball *et al.* (5)). The diphtheria bacillus, on the other hand, contains very little cytochrome *c* or cytochrome oxidase, and cytochrome *b* appears to be the limiting factor concerned in succinate oxidation by these organisms (2).

In the present study we have investigated the succinoxidase systems of beef heart muscle and of the diphtheria bacillus. The principal difference between the bacterial and mammalian systems resides in the relative amounts of the various components of the succinoxidase system present, as noted above. Of particular interest is the exceptionally high succindehydrogenase activity of the diphtheria bacillus, which parallels its high cytochrome *b* content. The evidence to be presented suggests that cytochrome *b* oxidation is the limiting factor in the oxidation of succinate by tissue and bacterial extracts in the presence of methylene blue and cyanide and that succindehydrogenase and cytochrome *b* may be identical both in diphtherial extracts and beef heart muscle succinoxidase preparations.

EXPERIMENTAL

Hemin Analyses—1 to 2 cc. of crude diphtherial sonic extract containing 6 to 8 mg. of nitrogen per cc. (2 to 3 γ of hemin iron per cc.) are treated

with 1 cc. of 0.2 N NaOH and 2 cc. of reagent grade pyridine. The volume is then made up to 10 cc. with distilled water. In the case of crude sonic extracts this solution is always somewhat cloudy and must be clarified by centrifugation at high speed (1 hour at 10,000 R.P.M. in the International refrigerated centrifuge). The clear straw-colored supernatant is placed in two cuvettes and a little sodium hydrosulfite added to one of them. The difference in absorption between oxidized and reduced pyridine hemochromogens at 554 mμ is then read in the Beckman spectrophotometer and compared with a standard pyridine hemochromogen containing 5.5 γ of hemin chloride per cc.

With crude bacterial extracts the results are fairly satisfactory, although there is some non-specific absorption even in the visible region. Moreover, the values are probably somewhat low because of some occlusion of hemochromogen in the precipitate. With purified cytochrome b preparations, however, no centrifugation is necessary, the 20 per cent pyridine solution is clear, and the absorption spectrum is superimposed on that of pure pyridine hemochromogen throughout the entire visible region. It must be realized that only the dissociable protohemin is determined by this method. The hemins of other iron enzymes, such as cytochrome c and catalase, do not form pyridine hemochromogen under these conditions.

The total iron content of diphtheria bacilli grown on a medium containing an excess of iron is about 0.85 γ per mg. of bacterial nitrogen (6). About 40 to 60 per cent of this iron can be accounted for as hemin iron by the above procedure.

Succindehydrogenase Determination—Succindehydrogenase activity was estimated in the Warburg apparatus at 36.35° in 0.1 M phosphate buffer at pH 7.8. Each vessel contained 0.2 cc. of 20 per cent NaOH in the center cup, 0.2 cc. of 0.16 M sodium succinate, 0.1 cc. of 0.02 M KCN, and an appropriate amount of enzyme.[1] After equilibration, 0.2 cc. of 0.1 per cent methylene blue was tipped in from the side arm. The total volume was 2 cc. per vessel. Q_{O_2} values were calculated from the oxygen uptake during the first 15 minutes and are expressed as c.mm. of O_2 per mg. of N per hour.

Succindehydrogenase activity was occasionally checked by the ferricyanide method of Quastel and Wheatley (7). The results were in good agreement with those by the methylene blue method; i.e., $Q_{CO_2}^{N_2}$ (ferricyanide) = $4Q_{O_2}$ (methylene blue).

Preparation of Crude Bacterial Extracts—The Toronto strain of *Corynebacterium diphtheriae* was grown in 5 to 10 liter lots on Mueller and Mil-

---

[1] Low Q_{O_2} values are obtained if too much enzyme is present, because the rate of oxidation of reduced methylene blue becomes limiting. For this reason, more than one enzyme concentration was usually tested.

ler's casein hydrolysate medium (8) containing 25 mg. of $FeSO_4 \cdot 7H_2O$ per liter. The organisms were harvested after 6 to 7 days growth at 34°, collected by centrifugation, and washed three times with saline. The final pH of the culture filtrate was usually 6.8 to 7.0. The washed organisms from 5 liters of culture were suspended in 150 to 200 cc. of saline and the suspension disrupted for 30 minutes in the 9000 cycle sonic oscillator[2] in 25 cc. portions. The final crude extract usually contained 6 to 8 mg. of nitrogen per cc. at this stage.

Purification of Diphtherial Cytochrome b and Succindehydrogenase Activity by Differential Centrifugation—The purification and concentration procedure varied somewhat from lot to lot. The procedure used to obtain a typical cytochrome b concentrate was as follows:

Preparation 21—Washed organisms from 5 liters of culture were suspended in 180 cc. of saline and disrupted in the sonic oscillator as described above. The crude extract contained 7.58 mg. of nitrogen per cc. and 0.3 mg. of hemin iron per mg. of nitrogen. The Q_{O_2} (succinate-KCN) and Q_{O_2} (succinate-methylene blue-KCN) were 146 and 4070 c.mm. per mg. of N per hour respectively. The crude extract was centrifuged for 30 minutes at 10,000 R.P.M. in a refrigerated International centrifuge. The turbid supernatant was removed from bacterial débris and centrifuged in a Sorvall vacuum type angle centrifuge, model SS-2, at 12,000 R.P.M. (about 18,000 $\times$ g) for 30 minutes. After centrifuging, three layers were present. About 25 cc. of a fatty yellow upper layer of low activity were removed and discarded. 130 cc. of a clear red layer, containing more than 60 per cent of the total activity (Fraction B), was next removed. Its nitrogen content was 4.41 mg. per cc. and the Q_{O_2} (succinate) was 140. The sediment (Fraction C), containing 30 per cent of the total activity, was discarded in this particular experiment. Fraction B was then centrifuged at 16,000 R.P.M. (about 30,000 $\times$ g) for 3 hours. Once again three layers were obtained: an inactive yellow topmost layer, a clear reddish brown layer with considerable activity, and a dark wine-red transparent sediment at the bottom of the tubes. About 30 per cent of the total original activity was contained in the clear red sediment after the supernatant was poured off. This sediment was suspended in 0.02 M phosphate buffer and homogenized in the sonic vibrator for 20 minutes. 35 cc. of a dark red solution were obtained, which was clarified by centrifugation in the model SS-2 Sorvall centrifuge for 30 minutes at 12,000 R.P.M. The sediment was discarded and the supernatant dark red solution (Fraction B3) was clear by transmitted light but showed a marked Tyndall effect. The preparation showed strong cytochrome b bands at 560 and 529 mμ after 1:5 dilution and reduction with hydrosulfite. Frac-

---

[2] Raytheon Corporation, Waltham, Massachusetts.

tion B3 contained 3.90 mg. of N per cc. and 1.09 γ of hemin iron per mg. of nitrogen. The Q_{O_2} (succinate) was 440 and Q_{O_2} (succinate-methylene blue) was 14,000 c.mm. of O_2 per hour per mg. of N. The purification was thus 3- to 3.5-fold both on the basis of activity and of hemin content.

Purified cytochrome b-succindehydrogenase preparations may be kept either in the frozen state at $-70°$ or may be lyophilized and stored in a desiccator over P_2O_5. The preparations keep for months without loss of activity, but reconstitution of the frozen or dried material yields solutions which are more turbid than the original preparation. Both cytochrome b and succindehydrogenase activity may be precipitated at pH 4.8, washed with water, and resuspended in phosphate buffer at pH 7.8 without loss of potency. Little additional purification is obtained by acid precipitation.

Heart Muscle Succinoxidase Preparations—Succinoxidase was prepared from beef heart by the method of Keilin and Hartree (9) except that the defatted, minced, washed muscle pulp was suspended in chilled phosphate buffer (pH 7.0) by means of a Waring blendor rather than by grinding with sand. The final precipitate obtained with acetate buffer of pH 4.5 was washed with chilled distilled water to remove excess cytochrome c before resuspending in 0.1 M phosphate buffer at pH 7.3. The sonic oscillator was used to obtain a uniform suspension. On reduction with hydrosulfite, the Keilin and Hartree preparation showed strong absorption bands characteristic of cytochromes a_3 and b. The cytochrome c band was faint or absent. In a typical preparation, the final succinoxidase suspension obtained from 400 gm. of pressed, washed muscle pulp measured 70 cc. It contained 4.00 mg. of nitrogen per cc.

Results

Succinoxidase Systems of Beef Heart Muscle and Corynebacterium Diphtheriae—In Table I, Columns 1 and 3, the succinoxidase activity of an unfractionated homogenate of fresh beef heart muscle is compared with that of a crude sonic extract from freshly harvested diphtheria bacilli. The Q_{O_2} (succinate) of the heart muscle homogenate is about twice that of the bacterial extract. Addition of excess cytochrome c doubles the rate of oxygen uptake by the heart muscle preparation but has little effect on the Q_{O_2} of the diphtherial system. 2.5×10^{-3} M cyanide inhibits oxygen consumption in heart muscle to the extent of 95 per cent but brings about only a 10 per cent reduction in the Q_{O_2} of the fresh diphtherial extract. The most striking differences between the mammalian and bacterial preparations are their relative "succindehydrogenase" contents as measured by the methylene blue method and cytochrome oxidase contents as measured by oxidation of p-phenylenediamine in the presence of excess cytochrome c. Succinate is oxidized more than 15 times as rapidly by the

diphtheria bacillus as by beef heart muscle in the presence of methylene blue and potassium cyanide. On the other hand, p-phenylenediamine is oxidized very slowly by the bacterial extracts as compared with its rapid oxidation by heart muscle.

These differences appear even more striking when the activity of purified diphtherial cytochrome b is compared with the Keilin and Hartree succinoxidase preparation. As is seen from Columns 2 and 4 of Table I, cytochrome c and KCN are without effect on the diphtherial preparation and cytochrome oxidase activity is completely absent. Addition of methylene blue (with or without cyanide) increases the Q_{O_2} more than 30-fold. The Keilin and Hartree preparation, on the other hand, shows a 5-fold increase in Q_{O_2} on adding excess cytochrome c and almost complete inhibition by 2.5×10^{-3} M KCN. Activity in the presence of cyanide is

TABLE I

Comparison between Activity of Diphtherial and Mammalian Succinoxidase Systems at pH 7.8

Substrate*	Q_{O_2}†			
	Beef heart homogenate	Keilin-Hartree preparation	Diphtheria bacillus sonic extact	Purified diphtherial cytochrome b
	(1)	(2)	(3)	(4)
Succinate.........................	312	220	162	440
" + cytochrome c.................	646	1100	168	440
" + KCN	14	10	146	440
" + " + methylene blue......	254	1500	4070	14,000
Phenylenediamine + cytochrome c	1470	6000	15	0

* Final concentration of succinate and phenylenediamine, 0.02 M; KCN, 10^{-3} M; cytochrome c, 5×10^{-5} M; methylene blue, 0.01 per cent.

† C.mm. of O_2 per mg. of N per hour calculated from the first 15 minutes.

restored by methylene blue but remains far inferior to that in the diphtherial extract. Phenylenediamine is oxidized several times as fast as is succinate.

Effect of pH on Oxidation of Succinate by Diphtherial Cytochrome b Preparations—It has been shown previously (2) that the rate-limiting step in the oxidation of succinate by diphtherial extracts in the presence of cyanide is the autoxidation of cytochrome b. The Q_{O_2} under these conditions is thus a measure of cytochrome b concentration.[3] The method is not a

[3] We previously stated (2) that when succinate is added to diphtherial extracts anaerobically 50 to 60 per cent reduction of cytochrome b occurs within a few minutes. This statement is misleading, since the reduction occurs almost instantaneously and the bands of reduced cytochrome b are immediately visible, even in the presence of air.

sensitive one, however, since reduced cytochrome b is oxidized at a relatively slow rate by molecular oxygen. Addition of an excess of methylene blue to diphtherial extracts or purified cytochrome b preparations increases the Q_{O_2} some 25- to 30-fold even in the presence of cyanide.

The Q_{O_2} under these conditions is generally considered to be a measure of succindehydrogenase activity. In Fig. 1 we have compared the succindehydrogenase activity measured in this way with the rate of oxidation
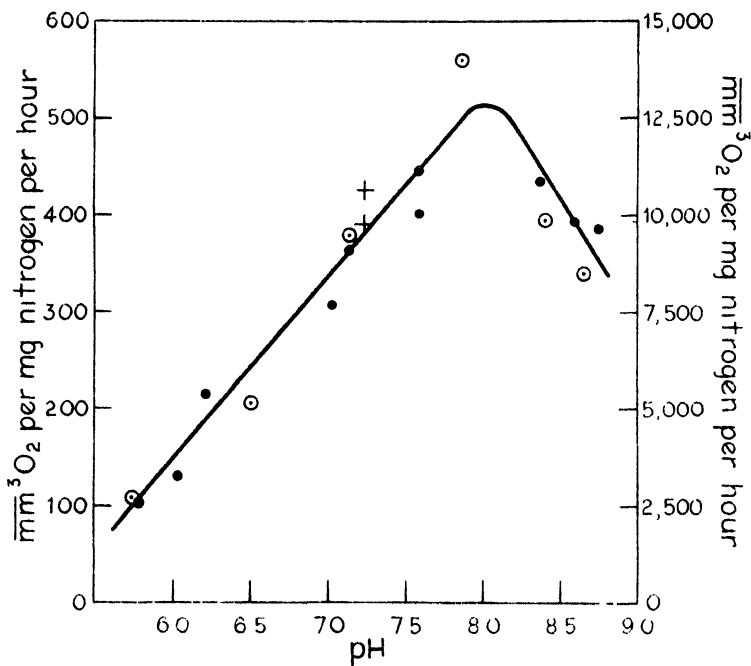
Fig. 1. Effect of pH on succinate oxidation by purified diphtherial cytochrome b (Preparation 21B3). ●, without added methylene blue; ⊙, methylene blue added; +, 0.25 $Q_{CO_2}^{N_2}$ (ferricyanide). pH 5.8 to 8 (0.1 M phosphate buffer); pH 8 to 9 (NaCl-borate buffer).

of cytochrome b by molecular oxygen in the pH range 5.8 to 8.6. In both cases the optimum pH is 7.8 to 8.0. Although the Q_{O_2} values at pH 5.8 are less than 25 per cent of the values at pH 7.8, the ratio of succindehydrogenase activity to cytochrome b autoxidation remains practically constant between 25:1 and 30:1. The results suggest that oxidation of cytochrome b is the rate-controlling step not only when molecular oxygen is the oxidant but also when oxygen is replaced by methylene blue.

Effect of pH on Succindehydrogenase Activity of Keilin and Hartree's

Preparation—As is shown in Table II, the optimum pH for oxidation of succinate by the Keilin and Hartree preparation in the presence of methylene blue and cyanide is also 7.8 to 8.0. The Q_{O_2} increases about 6-fold in the pH range 6 to 8. By analogy with the diphtherial system, it seems likely that here again oxidation of cytochrome b by methylene blue is the rate-controlling step. The Keilin-Hartree preparation differs from the bacterial preparation in that the former system is rapidly inactivated above pH 8. The Q_{O_2} at pH 8.5 and 8.8 was calculated from the oxygen consumption during the first 5 minutes after the enzyme was tipped in from the side arm of the vessel and cannot be regarded as significant.

Heat Inactivation of Succinoxidase Preparations—In a further attempt to differentiate between cytochrome b and succindehydrogenase activity, the effect of different temperatures on the enzymic activity of purified diph-

TABLE II

Effect of pH on Succindehydrogenase Activity of Keilin-Hartree Preparation

pH	Q_{O_2}* (methylene blue + KCN)
6.0	180, 254
6.5	370, 408
7.3	1270, 1350
7.7	1450, 1720
8.0	1580
8.5†	910, 945
8.8†	194, 347

* Figures are given for two successive experiments.

† Calculated from the first 5 minutes. Enzyme tipped in from the side arm of the Warburg vessel.

therial cytochrome b in phosphate buffer at pH 7.6 was studied. When Preparation 21B3 was heated for 30 minutes at 60° under these conditions, aggregation occurred and the solution became turbid, but actual coagulation did not occur below 75° and was not complete below 80°. As is shown in Table III, about three-fourths of the activity was lost after 30 minutes at 75°, and inactivation was practically complete at 80°. Once again it was found impossible to differentiate between cytochrome b and succindehydrogenase activity.

The effect of heat on the succindehydrogenase activity of the Keilin-Hartree preparation was irregular and seemed to depend somewhat on the purity of the preparation. In one case complete coagulation and inactivation occurred at 60°; in another some activity still remained after 30 minutes at 75°.

Reduction of Cytochrome c by Cytochrome b in Presence of Succinate—

When *purified* diphtherial cytochrome b^4 is added to a solution containing succinate and cytochrome c in excess, the cytochrome c is slowly reduced. This reduction may be followed at 550 mμ in the Beckman spectrophotometer, as shown in Fig. 2. Potassium cyanide and naphthoquinone SN-5949 (5) have no effect on the rate of reduction of cytochrome c by cytochrome b. The rate of reduction is very slow, however, and at pH 7.3 diphtherial cytochrome b is autoxidized faster than it is oxidized by cytochrome c. The turnover number for cytochrome c reduction at 25° under these conditions, calculated from the hemin iron content of purified cytochrome b, is only about 7. This may be compared with a turnover number of 1420 for added cytochrome c in the oxidation of succinate by the Keilin-Hartree preparation (10). In Table IV we have

<div align="center">TABLE III</div>

Effect of Heat on Succinate Oxidation by Purified Diphtherial Cytochrome b
(Preparation 21B3)

0.1 M phosphate buffer at pH 7 6.

Heated 30 min. at	Q_{O_2} (autoxidation of cytochrome b)	Q_{O_2} (methylene blue)	Appearance
°C.			
25	400	14,000	Almost clear
60	300	9,000	Turbid
65	235	15,000	"
70	264	11,600	"
75	95	3,900	Partial coagulation
80	5	240	Complete "

calculated the turnover numbers for diphtherial cytochrome b during the oxidation of succinate under various conditions.

From Fig. 2 it may be seen that cytochrome c was reduced about 60 times faster in the presence of succinate and cyanide by the Keilin-Hartree preparation than by the diphtherial system, despite the fact that the former preparation contained only 15 per cent as much succindehydrogenase activity. The mammalian preparation was almost completely inhibited by naphthoquinone SN-5949. Ball *et al.* (5) have suggested that SN-5949 blocks a factor which mediates the reduction of cytochrome c by cytochrome b. The existence of such a factor has also been suggested by Stoppani (11), Slater (12), and others. Because the bacterial cytochrome

---

[4] Crude sonic extracts from the diphtheria bacillus invariably contain a substance which reduces cytochrome c even in the absence of added substrate. This reducing substance is removed during purification by differential centrifugation or by precipitation of cytochrome b at pH 4.8.
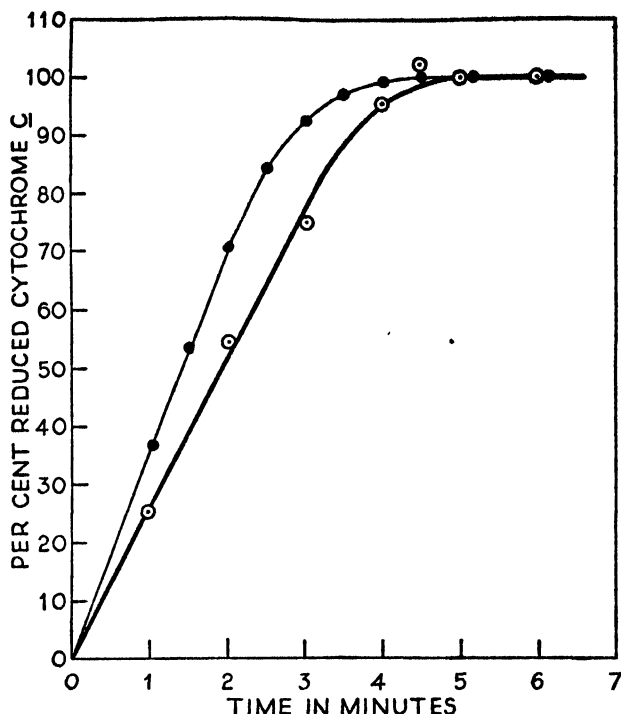
FIG. 2. Reduction of cytochrome c in the presence of succinate at 25°. One cuvette contained 3 cc. of 3×10^{-5} M cytochrome c and 0.01 M sodium succinate in 0.1 M phosphate buffer of pH 7.3 The control cuvette contained no succinate. 0.1 cc. of enzyme added to each cuvette and reduction followed at 550 mμ in the Beckman spectrophotometer. ●, 0.1 cc. of 1:50 Keilin-Hartree preparation, 5.37 mg. of N per cc. and Q_{O_2} 1500 in the presence of methylene blue and KCN   ⊙, 0.1 cc. of undiluted diphtherial cytochrome b (Preparation 2213) containing 5.96 mg. of N per cc. and Q_{O_2} 10,100 in the presence of methylene blue.

TABLE IV

Turnover Numbers* at 36.3° of Diphtherial Cytochrome b during Oxidation of Succinate under Varying Conditions

Oxidant	pH 7.25	pH 7.3
Methylene blue	1600	2120
Ferricyanide	1480	
O_2	56	66
Cytochrome c (25°)	7	

* Ratio of oxygen uptake per minute to oxygen equivalent of hemin iron added.

b preparation reduces cytochrome c so slowly, it should provide a good test system for the postulated factor acting between cytochromes b and

c in the succinoxidase system. We have tested various muscle, yeast, and bacterial extracts as well as a rabbit liver preparation prepared according to Stoppani (11) and a highly active preparation of cytochrome reductase prepared from *Escherichia coli*, but have been completely unsuccessful in finding any material which will accelerate the reduction of cytochrome *c* by diphtherial cytochrome *b* in the presence of succinate and potassium cyanide.

DISCUSSION

The succinoxidase system in cell-free extracts of beef heart muscle, which oxidizes succinic acid to fumaric acid, is a complex one which is not as yet fully understood. Of the various carrier systems involved in the transfer of 2 hydrogen atoms from succinate to oxygen, only cytochrome *c* has been isolated as a soluble pure protein. The complete succinoxidase system is generally supposed to consist of the following steps.

$$\text{Succinate} \xrightarrow{(1)} \text{succindehydrogenase} \xrightarrow{(2)} \text{cytochrome } b \xrightarrow{(3)}$$
$$\text{cytochrome } c \xrightarrow{(4)} \text{cytochrome oxidase } (a_3) \xrightarrow{(5)} O_2$$
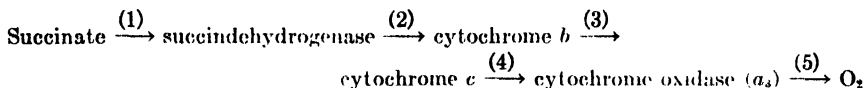
There is evidence that a factor exists between cytochromes *b* and *c*, and it has been suggested that cytochrome *a* may act between cytochromes *c* and oxidase (a_3). On the other hand, there is little clear-cut evidence that succindehydrogenase and cytochrome *b* exist as separate and distinct enzymes. The arrows indicate the direction of electron transfer and of increasing oxidation-reduction potential.

With the exception of cytochrome *c*, none of the components of the succinoxidase system has been separated from insoluble particles or from one another and much of the evidence for the various steps is derived from the action of inhibitors. However, the system can be simplified somewhat, since there are a number of substrates, such as *p*-phenylenediamine, cysteine, etc., which reduce cytochrome *c* directly. With *p*-phenylenediamine as substrate, for example, Steps 1 to 3 in the above scheme may be eliminated. In a similar way, the diphtherial system should prove useful for studying Steps 1 and 2, since, as we have shown (see Table I), purified diphtherial succinoxidase preparations are characterized by an exceptionally high succindehydrogenase activity and a very high cytochrome *b* content, but contain neither cytochrome oxidase nor cytochrome *c*.

Our attempts to separate diphtherial cytochrome *b* from the succindehydrogenase activity in bacterial extracts have been wholly unsuccessful. Both factors can be precipitated at pH 5 and cannot be separated by differential centrifugation. It was demonstrated previously (6) that the cellular iron content of the Toronto strain of *Corynebacterium diph-*

theriae can be varied over a 5- to 6-fold range by controlling the iron concentration of the media on which the organisms are grown. It has since been found that both succindehydrogenase activity and cytochrome *b* content are directly proportional to the iron content of the cells.

There can no longer be any doubt that the rate of succinate oxidation by the intact diphtheria bacillus is mainly determined by the rate of autoxidation of cytochrome *b*. With purified cytochrome *b* preparations, its autoxidation is the sole limiting factor, since the reduction of cytochrome *b* in the presence of succinate is practically instantaneous and no cyto-chrome oxidase is present. Although it appears likely that reduced diph-therial cytochrome *b* is oxidized by molecular oxygen at a somewhat higher rate than the cytochrome *b* of beef heart muscle (perhaps 2 to 3 times), its rate of autoxidation is extremely slow when compared with that of cytochrome oxidase. It is for this reason that the diphtheria bacillus requires such a high concentration of cytochrome *b*.

In the presence of methylene blue or ferricyanide, the rate of succinate oxidation is increased 25- to 30-fold. The Q_{O_2} (succinate) in the presence of methylene blue and cyanide or the $Q_{CO_2}^N$ in the presence of ferricyanide has generally been considered a measure of succindehydrogenase activity. However, as we have shown, succindehydrogenase activity measured in this way is always directly proportional to cytochrome *b* content, whether the latter is measured spectroscopically by its extinction at 560 mμ, by its rate of autoxidation in the presence of succinate and cyanide, or even by the slow rate of reduction of cytochrome *c* in the presence of succinate. It seems quite possible that succindehydrogenase is a hemin-containing enzyme which is in fact identical with cytochrome *b*. We are perhaps deal-ing with a system analogous to the lactic acid dehydrogenase from Delft yeast which Bach *et al.* (13) believe to be cytochrome b_2.

In an attempt to throw further light on the problem of whether one or two steps are involved in the oxidation of succinate by cytochrome *b*, we have studied the effect of pH on the system. As might be expected, the oxidation of succinate is very much slower at acid pH. Within the limits of experimental error, the ratio of Q_{O_2} (succinate-methylene blue)/Q_{O_2} (autoxidation of cytochrome *b*) or of $0.25Q_{CO_2}^N$ (succinate-ferricyanide)/Q_{O_2} (autoxidation of cytochrome *b*) is constant, *i.e.* about 25:1, at all pH values between 6 and 9. Optimum activity occurs at about pH 8 in all cases.[5] The effect of pH on succinate oxidation by the Keilin-Hartree preparation in the presence of methylene blue and cyanide is similar, and again the maximum Q_{O_2} is obtained at about pH 8. At more alkaline pH values the Q_{O_2} of the heart muscle preparation falls off more rapidly than with the

[5] In 1931, Cook and Alcock (14) found that succindehydrogenase activity of *E. coli* was optimum between pH 7.6 and 8.0 by the Thunberg technique.

diphtherial system. Keilin and Hartree (10) have shown that exposure of their preparation to pH 9 for 1 hour at room temperature results in complete loss of succindehydrogenase activity, while leaving the cytochrome oxidase system intact. At Dr. Keilin's suggestion, we have attempted to reactivate a succinoxidase preparation from beef heart, inactivated at pH 9, by addition of small amounts of diphtherial cytochrome b. No reactivation could be obtained. Apparently, the factor concerned in mediating the reduction of cytochrome c by cytochrome b is destroyed simultaneously with the succindehydrogenase activity at pH 9. The diphtherial system is stable even in the presence of the Keilin-Hartree preparation at pH 9.

Diphtherial cytochrome b is relatively heat-stable and purified preparations are only partially destroyed after 30 minutes at 75°. After $\frac{1}{2}$ hour at this temperature, 75 per cent of the activity against succinate is lost, whether measured by the rate of autoxidation of cytochrome b or in the presence of methylene blue.

In conclusion, it is our opinion that succindehydrogenase *may* well be identical with cytochrome b both in diphtherial extracts and in beef heart muscle succinoxidase preparations. Alternatively, succindehydrogenase and cytochrome b may be "intimately bound to the protein of a single colloidal particle forming thus a unit of a complete oxidizing system," as suggested by Keilin and Harpley (15) in considering the dehydrogenase-cytochrome system of *Escherichia coli*. In any event, it seems clear that by the usual methods for determination of succindehydrogenase, whether by oxygen uptake in the presence of methylene blue and cyanide or anaerobically with ferricyanide (Quastel and Wheatley (7)), *cytochrome b activity is actually measured*. If a true succindehydrogenase exists, separate and distinct from cytochrome b, which mediates the oxidation of succinic acid by cytochrome b, it must always be present in excess and so cannot be the rate-controlling step in the methods which have been used for its determination. In a recent note Slater (16) has arrived at a similar conclusion. From Slater's cytochrome b hemin analyses and succindehydrogenase (Q_{O_2}) determinations in heart muscle, a turnover number of 1120 per iron atom per minute may be calculated. This is in good agreement with our estimate of 1300 for the turnover number of diphtherial cytochrome b at pH 7.0 in the presence of methylene blue.

SUMMARY

The succinoxidase system of beef heart muscle has been compared with that of the diphtheria bacillus. The principal differences between the mammalian and bacterial systems reside in the relative amounts of the various cytochrome components present. Thus, beef heart muscle con-

tains relatively large amounts of cytochrome c, cytochrome oxidase, and the factor which mediates reduction of cytochrome c by cytochrome b. The diphtheria bacillus possesses far more succindehydrogenase activity and cytochrome b than does beef heart. On the other hand, the bacteria only possess traces of cytochrome c and cytochrome oxidase.

The evidence presented indicates that oxidation of cytochrome b is the rate-limiting step when succindehydrogenase activity is measured by oxygen uptake in the presence of methylene blue and potassium cyanide or by anaerobic oxidation with ferricyanide according to the method of Quastel and Wheatley.

BIBLIOGRAPHY

1. Fujita, A., and Kodama, T., *Biochem. Z.*, **273**, 186 (1934).
2. Pappenheimer, A. M., Jr., and Hendee, E. D., *J. Biol. Chem.*, **171**, 701 (1947).
3. Rawlinson, W. A., and Hale, J. H., *Biochem. J.*, **42**, p. xlvii (1948).
4. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **127**, 167 (1939).
5. Ball, E. G., Anfinsen, C. B., and Cooper, O., *J. Biol. Chem.*, **168**, 257 (1947).
6. Pappenheimer, A. M., Jr., *J. Biol. Chem.*, **167**, 251 (1947).
7. Quastel, J. H., and Wheatley, A. H. M., *Biochem. J.*, **32**, 936 (1938).
8. Mueller, J. H., and Miller, P., *J. Immunol.*, **40**, 21 (1941).
9. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **125**, 171 (1938).
10. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **129**, 277 (1940).
11. Stoppani, A. O. M., *Nature*, **160**, 52 (1947).
12. Slater, E. C., *Nature*, **161**, 405 (1948).
13. Bach, S. J., Dixon, M., and Zerfas, L. G., *Biochem. J.*, **40**, 229 (1946).
14. Cook, R. P., and Alcock, R. S., *Biochem. J.*, **25**, 523 (1931).
15. Keilin, D., and Harpley, C. H., *Biochem. J.*, **35**, 688 (1941).
16. Slater, E. C., *Biochem. J.*, **43**, p. li (1948).

VITAMIN E POTENCY OF α-TOCOPHEROL AND α-TOCOPHEROL ESTERS*

By PHILIP L. HARRIS and MARION I. LUDWIG

(*From the Research Laboratories, Distillation Products, Inc., Rochester, New York*)

Occasional vitamin E bioassays conducted in this laboratory prior to 1943 indicated that esterified tocopherols were more active biologically than equivalent quantities of the free tocopherols. Demole *et al.* (1) had also observed a similar difference in activity. However, in 1943 we stated that the observed differences were probably within the limits of uncertainty of the bioassay procedure and therefore were not significant (2). Now as the result of further study, a definite difference has been established.

Under standard test conditions (3) tocopherol esters, both natural and synthetic, showed a consistent superiority in vitamin E potency compared with the respective tocopherols in free alcohol form.

Results

Data obtained from ten bioassays, comparing the vitamin E potency of α-tocopherol esters with that of free α-tocopherol, are shown in Table I. The technique employed was that described by Mason and Harris (3) in which prevention of fetal resorption in rats is used as the criterion of biological response.

Esterified tocopherols were consistently more active than equivalent quantities of free tocopherol. This superiority in potency ranged from 10 to 127 per cent. In spite of the relatively great variation, the potency ratios were found to be a homogeneous group according to a χ^2 test (4). Consequently, a weighted mean was calculated and found to be 1.62. The standard error of this mean was 0.09. The probability is therefore 2 out of 3 that a similar group of bioassays would have a weighted mean of between 1.53 and 1.71.

It should be noted that this ratio between the potency of tocopherol and tocopherol ester holds both for natural and for synthetic α-tocopherol.

DISCUSSION

Why is α-tocopherol less potent in the free form than as an ester? Fat-soluble vitamins in general follow no such rule. Vitamin D in free form is more active physiologically than esterified vitamin D (5). Vitamin A

---

* Communication No. 147.

TABLE I

Relative Vitamin E Potency of α-Tocopherol and α-Tocopherol Esters

Thirty-six rats were used in each bioassay.

Assay date	Median fertility dose		Ratio $\dfrac{\text{M. F. D. }\alpha\text{-tocopherol}}{\text{M. F. D. }\alpha\text{-tocopherol as ester}}$	± s.e. of ratio†
	α-Tocopherol	α-Tocopherol as ester*		
Natural d,α-tocopherol and esters				
1947	mg.	mg		
Jan.	0.92	0.72	1.28	0.15
Feb.	0.60	0.43	1.40	0.27
"	0.44	0.40	1.10	0.40
Apr.	0.84	0.37	2.27	0.87
June	0.83	0.40	2.08	0.35
July	0.62	0.43	1.44	0.25
Oct.	0.64	0.34	1.88	0.34
Synthetic dl,α-tocopherol and esters				
Jan.	‡	0.67		
Feb.	0.81	0.52	1.56	0.33
"	0.60	0.41	1.46	0.45
Apr.	0.71	0.42	1.69	0.50
June	‡	0.61		
Weighted mean ± s.e.$_{WM}$§			1.62 ± 0.09	

* All samples of natural esters were pure d,α-tocopheryl acid succinate. All samples of synthetic esters were dl,α-tocopheryl acetate (international standard). The weights of tocopherol were calculated stoichiometrically from the weights of the esters.

† Standard error of the ratio, or of the comparison, of M. F. D. values =

$$\sqrt{\left(\frac{\text{s.e.}_F}{\text{M. F. D.}_F}\right)^2 + \left(\frac{\text{s.e.}_E}{\text{M. F. D.}_E}\right)^2}$$

s.e.$_F$ and s.e.$_E$ are standard errors of the mean fertility dose values of free α-tocopherol and of esterified tocopherol, respectively, and are calculated from dose-response curves according to Miller and Tainter (10).

‡ All test animals showed negative response at the 1.0 mg. level, which precluded calculation of M. F. D. values.

§ Weighted mean = $\Sigma(W_R \times \text{ratio})/\Sigma(W_R)$, where $W_R = 1/(\text{s.e. of ratio})^2$ (Miller *et al.* (4)). s.e. of the weighted mean = $\sqrt{1/\Sigma(W_R)}$ (Miller *et al.* (4)).

esters are superior to free vitamin A in bioassay tests (6),[1] although there is some evidence that dietary changes, such as increased fat intake, reverse this superiority (7).

[1] Harris, P. L., and Brinkman, J., unpublished data.

We tentatively explain our results on the basis that tocopherol esters are relatively more resistant than free tocopherol to oxidative destruction, such as might occur prior to absorption in the intestinal tract of the assay rats. Results of a few experiments indicate that by reducing the possibilities for intestinal destruction, *e.g.* adding antioxidants to the tocopherol solutions fed and to the diet, free α-tocopherol was preserved sufficiently to induce a response equivalent to that of an equimolar quantity of esterified α-tocopherol. Diamylhydroquinone fed in 15 mg. quantities simultaneously with free α-tocopherol supplements in several vitamin E bioassays significantly increased the effectiveness of the tocopherol as shown by an average reduction of the mean fertility dose from 0.67 mg. to less than 0.3 mg. However, as shown in Table I, α-tocopherol esters under standard bioassay conditions possess about 62 per cent more vitamin E potency than free α-tocopherol.

TABLE II

Biological Equivalency of Various Forms of α-Tocopherol

Form of tocopherol	Equivalency	Calculation
	I.U. per mg.	
dl, α-Tocopheryl acetate	1.00	By definition of I.U.
dl, α-Tocopherol.	0.68	$1.00 \div 0.91 \div 1.62 = 0.68$*
d, α-Tocopheryl acetate	1.36	Harris and Ludwig (9)
succinate	1.21	$1.36 \times 0.89 = 1.21$†
d, α-Tocopherol	0.92	$1.36 \div 0.91 \div 1.62 = 0.92$

* 0.91 = the ratio of the molecular weight of α-tocopherol to that of α-tocopheryl acetate; 1.62 = the ratio of the activity of α-tocopherol, as an ester, to that of α-tocopherol (Table I).

† 0.89 = the ratio of the molecular weight of α-tocopheryl acetate to α-tocopheryl succinate.

Biological equivalencies in terms of international units can now be assigned to the various forms of α-tocopherol. Starting with the defined potency of synthetic *dl*,α-tocopheryl acetate, 1 mg. = 1.00 I.U. (8), and the experimentally determined equivalency of natural *d*, α-tocopheryl acetate, 1 mg. = 1.36 I.U. (9), the values in Table II can be calculated.

SUMMARY

A comparison of the vitamin E potency of both natural and synthetic free α-tocopherol with esterified α-tocopherol was made by standard bioassay procedures.

α-Tocopherol, in the form of an ester, showed 62 per cent more vitamin E potency than did free α-tocopherol. This relationship was found both for natural tocopherols, *d*,α esterified *versus* *d*,α free, and for synthetic tocopherols, *dl*,α esterified *versus* *dl*,α free.

Biological equivalencies were calculated for α-tocopherol in the free form dl,α-tocopherol, free $\cong$ 0.68 I.U. per mg.; d,α-tocopherol, free $\cong$ 0.92 I.U. per mg.

BIBLIOGRAPHY

1. Demole, V., Isler, O., Ringier, B. II., Salomon, H., and Karrer, P., *Helv. chim. acta*, **22**, 65 (1939).
2. Joffe, M., and Harris, P. L., *J. Am. Chem. Soc.*, **65**, 925 (1943).
3. Mason, K. E., and Harris, P. L., *Biol. Symposia*, **12**, 459 (1947).
4. Miller, L. C., Bliss, C. I., and Braun, II. A., *J. Am. Pharm. Assn.*, **28**, 644 (1939).
5. Bailey, B. E., and Green, K., *Fisheries Res. Board Canada, Progress Rep.*, *No. 47*, 12 (1941).
6. Moll, T., and Reid, A., *Z. physiol. Chem.*, **260**, 9 (1939).
7. Week, E. F., Presented before the Animal Nutrition Research Council, Washington, Oct. 13 (1948).
8. Hume, E. M., *Nature*, **148**, 472 (1941).
9. Harris, P. L., and Ludwig, M. I., *J. Biol. Chem.*, **179**, 1111 (1949).
10. Miller, L. C , and Tainter, M. L., *Proc. Soc. Exp. Biol. and Med.*, **57**, 261 (1944)

CHOLESTEROL ESTERASES

I. PREPARATION OF SUBSTRATE MIXTURE AND CHARACTERIZATION OF THE HYDROLYTIC CHOLESTEROL ESTERASE OF PANCREATIN*

By RICHARD S. YAMAMOTO, NORMAN P. GOLDSTEIN, AND C. R. TREADWELL

(From the Department of Biochemistry, School of Medicine, George Washington University, Washington)

The enzyme or enzymes involved in the formation and hydrolysis of cholesterol esters have been the subject of sporadic studies since 1910 when Kondo (1) reported that extracts of horse and ox liver catalyzed the hydrolysis of these esters. The most extensive studies have been carried out by Sperry and coworkers (2) who also reviewed the earlier literature and pointed out the lack of agreement between several of the reports. A critical review of the previous work will show the following points which are of interest in connection with the studies on cholesterol esterase in progress in our laboratory. It has not been clearly shown whether the synthesis and hydrolysis of cholesterol esters are catalyzed by a single enzyme or by different enzymes. The enzyme activities related to cholesterol esters have not been definitely distinguished from the activity of other esterases occurring in the same tissues. The identity of the enzymes reported in various tissues such as liver, blood, and pancreas has not been established. There has not been an adequate characterization of the enzyme from any of the reported sources.

In a majority of the studies serum has been used to supply the substrate. This has been unfortunate for several reasons. This substrate mixture contains the end-products of the reaction irrespective of whether the synthesizing or hydrolyzing activity is under study. Also this substrate mixture has been reported to possess both kinds of activity, depending on the experimental conditions. Moreover, the use of serum limits the types of controls and experimental conditions which may be employed. In general the studies with serum have suggested a low order of activity for the enzyme; in many cases the incubation time has been extended to 48 or 72 hours in order to demonstrate appreciable synthesis or hydrolysis. The small amount of substrate, usually of the order of 1 mg. or less, has also introduced the possibility of considerable error in apparent activity due to small errors in the determination of cholesterol.

Since the experiments described below were completed, Nieft and Deuel (3) and Nieft (4) have reported studies on the cholesterol esterases of rat liver and intestine. The existence of enzyme systems in both organs for synthesizing and hydrolyzing cholesterol esters has been verified. The esterifying system requires the presence of phosphate ion and a fatty acid source. The activity of the hydrolytic system was accentuated by the presence of soya lecithin. Also the hydrolytic system of rat intestine was found to consist of at least two factors.

In planning a detailed and extensive study of the synthesis and hydrolysis of cholesterol esters it seemed necessary first to devise substrate mixtures which could be employed in a variety of different types of experiments, and secondly, to characterize the catalytic activity from some easily obtainable standard source to provide a basis for assaying various tissues and fluids for activity and for comparing the activity from various sources. The present report presents our data on a suitable substrate mixture for studying the enzymatic hydrolysis of cholesterol esters and a characterization of the hydrolytic activity of a commercial pancreatin.

EXPERIMENTAL

Preparation of Cholesterol Oleate—The ester was prepared according to the directions of Page and Rudy (5). The product, which was obtained in 85 per cent yield, melted at 44.0° and gave no precipitate with digitonin. For ease in handling it was stored in a vacuum desiccator in the refrigerator, for at room temperature the crystals gradually became amorphous and sticky.

Preparation of Substrate Mixture—A weighed amount (100 mg. or less) of cholesterol oleate is placed in each of a series of test-tubes (25 × 200 mm.), previously tested for use with a stainless steel pestle of the Potter-Elvehjem homogenizing apparatus (6), and 0.5 cc. of ethyl ether is added to dissolve the ester. The following reagents are then added to each tube in order: 1 cc. of 10 per cent sodium glycocholate, 10 cc. of 0.154 M potassium phosphate buffer, 1 cc. of 1:1000 merthiolate, and 500 mg. of egg albumin, impalpable powder, soluble. The contents of the tubes are then homogenized for 1 minute. Following this the tubes are placed in a specially designed shaking apparatus at 37° for 1 hour. The tubes are moved through a vertical arc of approximately 55° at a rate of 100 excursions per minute while immersed in a constant temperature bath. After the preliminary shaking period which brings the temperature of the substrate mixture to that of the bath and removes the ether, the tubes are removed and 1 cc. of enzyme solution or substitute is added to each tube. The contents are mixed well by hand and two samples (1 cc. or less) are removed for determination of zero hour values. The tubes are immediately

returned to the shaker. The volume of solution in the tubes is not allowed to fall below 7 cc. during an experiment; so when it is necessary to remove more than 8 cc. of samples the amounts of all the constituents are doubled.

We routinely determined both the total and free cholesterol content of the samples by the method of Schoenheimer and Sperry (7) with the modification of Sperry (8). The determination of both fractions of cholesterol allowed for calculations of the per cent hydrolysis of the ester on the basis of the increase in free cholesterol or the decrease in cholesterol ester and in addition provided data (total cholesterol content) for evaluation of the uniformity of the emulsion during incubation. Substrate mixtures prepared as described above and shaken for the duration of an experiment were uniform in total cholesterol content up to 48 hours. In general the total cholesterol content of aliquots did not vary more than

TABLE I

Total Cholesterol Content of Typical Digests During Incubation

The digests were prepared as described in the text. pH of digest, 6.6.

Digest No.	Total cholesterol during incubation					
	0 hr.	3 hrs.	12 hrs.	24 hrs.	Average	Variation
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
1	16.0	16.6	16.9	15.9	16.4	±3.0
2	15.7	16.3	17.1	16.8	16.5	±4.8
3	49.7	48.8	49.3	50.7	49.6	±2.2
4	50.4	48.4	51.2	49.9	50.0	±3.2

duplicate analyses on the same aliquot. This result indicates that, within the limits of accuracy of the method used for determining the cholesterol, the mixtures are uniform in composition throughout the duration of an experiment. Occasionally, the total cholesterol content of a single sample differed markedly from the average of the others obtained from the same digest. These were very likely due to sampling errors and were rejected from the calculations. The total cholesterol content of samples removed after 0, 3, 12, and 24 hours of incubation of representative digests is given, in Table I.

During the development of the substrate mixture a large number of emulsifying agents were tested singly and in various combinations. Sodium glycocholate and egg albumin were the only combination tested which produced a stable and uniform emulsion of the ester. When 1 cc, of sodium glycocholate solutions of concentrations less than 10 per cent was employed, the total cholesterol content of serial samples indicated that,

the ester was not uniformly dispersed. While such preparations could be used to demonstrate the esterase qualitatively, they were obviously unsuitable for quantitative serial studies. On the basis of these findings 1 cc. of 10 per cent sodium glycocholate was adopted as an essential part of the substrate mixture. It should be emphasized that the results obtained with this substrate mixture only define the activity of the enzyme in the presence of this concentration of sodium glycocholate. Sperry and Stoyanoff (9) have shown that the effect of sodium glycocholate on cholesterol esterase varies with the concentration of bile salt and the source of the enzyme. They concluded that the correct explanation for the effects of bile salts must await further experimentation. Thus, while the sodium glycocholate functioned as an emulsifying agent in the preparation of the substrate mixture, it is possible that during incubation it had a secondary effect on the activity of the enzyme. However, this secondary effect, if present, was a constant in all of the experiments reported below.

TABLE II

Determination of Inactivation Temperature of Cholesterol Esterase of Pancreatin

The digests were prepared as described in the text. Cholesterol oleate, 100 mg.; pH 6.6. Incubation time, 24 hours. Portions of a 20 per cent pancreatin solution were heated in a water bath for 1 hour at the temperatures designated. 1 cc. of heated solutions added to digest.

Digest No.	1	2	3	4	5	6
Temperature, °C.	37	50	55	60	65	70
Hydrolysis, %	37.7	30.0	17.7	11.3	0.1	0.0

In preliminary experiments with the substrate mixture, control digests containing 1 cc. of water or of boiled pancreatin solution were incubated concurrently with active digests. These consistently showed no hydrolysis and were omitted in later experiments.

Characterization of Hydrolytic Cholesterol Esterase of Pancreatin—The pancreatin, U. S. P., Merck, was suspended in a glycerol-water mixture (1:1) to give a 20 per cent concentration, except in Experiment IV on the effect of the concentration of enzyme.

Experiment I; Inactivation Temperature—Shope (10) reported that the temperature at which the enzyme is inactivated lies somewhere below 67°, and that boiling for 5 minutes completely destroyed the activity. Sperry and Stoyanoff (9) incubated serum for 1 hour at 55–60° to inactivate the enzymes before using it as substrate. In the present experiment portions of the 20 per cent pancreatin suspension were heated for 1 hour at the temperatures designated in Table II, and then 1 cc. samples were added to the substrate mixture and incubated as usual. The data in Table II

show that the enzyme responsible for the hydrolytic activity was inactivated by heating for 1 hour at 65°. In several experiments not reported here, the activity was completely destroyed by placing the pancreatin solution in a boiling water bath for 15 minutes.

Experiment II; Determination of Optimum pH for Hydrolytic Activity—Using serum as a substrate, Klein (11) reported two types of hydrolyzing cholesterol esterases. One type found in liver, spleen, kidney, and intestinal mucosa had an optimum pH of 5.3. The other was found in pancreas and was active over a pH range of 5.6 to 9.2, with an optimum range from pH 6.3 to 8.7. Recently, Le Breton and Pantaleon (12) reported that dog pancreas was active at pH 5.3 and at 7.3.

TABLE III

Influence of pH on Hydrolysis of Cholesterol Oleate by Pancreatin

The digests were prepared as described in the text. Cholesterol oleate, 100 mg. Enzyme, 1 cc. of 20 per cent pancreatin. Time, 24 hours.

Digest No	pH			Hydrolysis
	Original buffer	Digests		
		0 hr.	24 hrs.	
				per cent
1	4.0*	3.35	3.55	2.7
2	4.0	5.25	5.32	20.5
3	5.0	5.33	5.37	20.6
4	6.0	5.73	5.74	21.8
5	7.0	6.64	6.40	36.2
6	8.0	7.11	6.98	19.2
7	9.0	7.35	7.00	16.3
8	9.0*	9.60	7.97	0.9

* See Experiment II in the text.

A series of potassium phosphate buffers (0.154 M) in intervals of 1 pH unit from pH 4 to 9 was used in preparing the substrate mixtures. For the extreme values hydrochloric acid and sodium hydroxide were added to substrate mixtures containing the buffers of pH 4 and 9 respectively. The data, shown in Table III, indicate that the enzyme has a sharp maximum for hydrolytic activity at approximately pH 6.6. In this experiment and in additional runs there was no indication of a second optimum pH for the hydrolytic activity.

Experiment III; Time-Rate Relationship and Effect of Substrate Concentration—Fig. 1 shows the time-rate curves for three concentrations of substrate. The curves for the two higher concentrations approach those for a first order reaction. The curve for 33 mg. of substrate is intermediate between those for zero order and first order reactions. Comparable data

were also obtained with 2.5, 5, and 10 per cent pancreatin solutions. In general the specific reaction rate decreased with time at all substrate and enzyme concentrations studied.

Experiment IV; Effect of Concentration of Enzyme—A 20 per cent solution of pancreatin was diluted with a glycerol-water mixture (1:1) to give concentrations of 2.5, 5, and 10 per cent. The results shown in Fig. 2 were obtained with 100 mg. of substrate per digest. The percentage hydrolysis was not strictly proportional to the enzyme concentration over the range studied. There was a decrease in the percentage hydrolysis
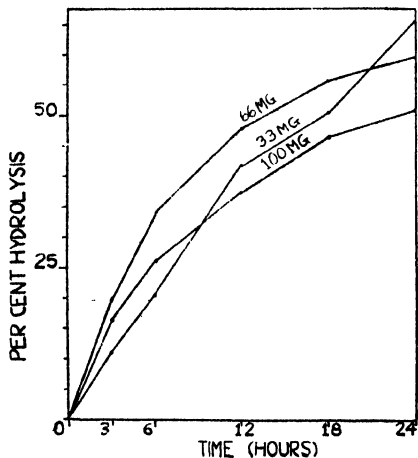
[Fig. 1
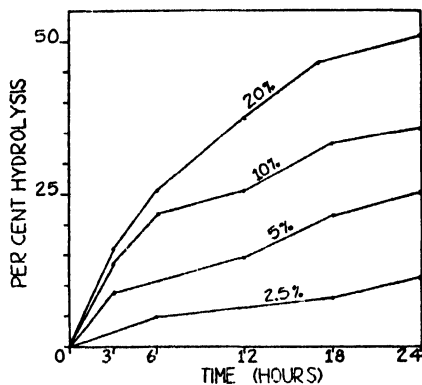
Fig. 2

FIG. 1. Time-rate relationship and effect of substrate concentration. The digests were prepared as described in the text. pH of digests, 6.6. Enzyme, 1 cc. of 20 per cent pancreatin. The amount of cholesterol oleate per digest is indicated on the curves.

FIG. 2. Effect of concentration of enzyme. The digests were prepared as described in the text. Cholesterol oleate, 100 mg. pH of digests, 6.6. The concentrations of pancreatin indicated on the curves were prepared by dilution of the 20 per cent pancreatin with a glycerol-water mixture (1:1).

per unit amount of pancreatin as the concentration of enzyme increased. This is a common finding with crude enzyme preparations as used in these experiments. However, the data suggest that under specific conditions of substrate concentration, pH, and incubation time a quantitative assay of the enzyme activity will be possible.

SUMMARY

The preparation of a cholesterol oleate substrate mixture and standard conditions suitable for studying the cholesterol ester-hydrolyzing activity

of tissues and fluids are described. The substrate mixture was used in studying the following characteristics of the hydrolytic activity of a commercial pancreatin. The enzyme was inactivated by boiling for 15 minutes or heating for 1 hour at 65°. The optimum pH was approximately 6.6. The time-rate relationships during a 24 hour incubation period for a series of substrate and enzyme concentrations were determined.

BIBLIOGRAPHY

1. Kondo, K., *Biochem. Z.*, **26**, 243 (1910); **27**, 427 (1910).
2. Sperry, W. M., and Brand, F. C., *J. Biol. Chem.*, **137**, 377 (1941).
3. Nieft, M. L., and Deuel, H. J., Jr., *J. Biol. Chem.*, **177**, 143 (1949).
4. Nieft, M. L., *J. Biol. Chem.*, **177**, 151 (1949).   •
5. Page, I. H., and Rudy, H., *Biochem. Z.*, **220**, 304 (1930).
6. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
7. Schoenheimer, R., and Sperry, W. M., *J. Biol. Chem.*, **106**, 745 (1934).
8. Sperry, W. M., *Am. J. Clin. Path.*, **8**, *Tech. Suppl.*, **2**, 91 (1938).
9. Sperry, W. M., and Stoyanoff, V. A., *J. Biol. Chem.*, **117**, 525 (1937).
10. Shope, R. E., *J. Biol. Chem.*, **80**, 127 (1928).
11. Klein, W., *Z. physiol. Chem.*, **254**, 1 (1938); **259**, 268 (1939).
12. Le Breton, E., and Pantaleon, J., *Arch. sc. physiol.*, **1**, 63 (1947).

ACETYLATION IN THE DIABETIC RAT*

By FRIXOS C. CHARALAMPOUS and D. MARK HEGSTED

(From the Department of Nutrition, Harvard School of Public Health, and the Department of Biological Chemistry, Harvard Medical School, Boston)

The mechanism of metabolic acetylations has been studied by many investigators. Lipmann and his coworkers (1) demonstrated that the enzyme system involved in this reaction requires coenzyme A, a pantothenic acid derivative. The indispensability of pantothenic acid for normal acetylation *in vivo* was shown in this laboratory with deficient rats (2). The decrease in the acetylation of a dose of *p*-aminobenzoic acid which is found in pantothenic acid-deficient as compared to normal animals is presumably due to a decrease in the coenzyme A content of the tissues (3). However, deficiencies of thiamine and riboflavin also resulted in some decrease in the extent of acetylation of aromatic amines which did not respond to the administration of pantothenic acid or additional acetate (4). The coenzyme content of various tissues from riboflavin-deficient animals was not significantly different from that of non-deficient controls (3). To explain these results a deficiency of other components of the enzyme system must be implicated, such as the enzyme itself or the energy supply required for the reaction.

Klein and Harris (5) showed an energetic coupling between acetylation and respiration, since acetylation of sulfanilamide by rabbit liver slices was greatly decreased anaerobically. The addition of acetate, acetaldehyde, and pyruvate increased acetylation to various degrees. These results were interpreted as effects upon acetate formation.

Lipmann (6, 7) subsequently demonstrated that enzymic acetylation of aromatic amines depends on the availability of high phosphate bond energy. Adenyl pyrophosphate was found to replace the respiratory reactions in pigeon liver homogenates on which acetylation depended. Similarly, the acetylation of choline by brain slices or homogenates depends on the availability of high phosphate bond energy (8–10).

The results presented in this paper indicate (*a*) that the acetylation of alloxan-diabetic rats is less than normal and (*b*) that the defect is apparently due to a relative deficiency of adenosine triphosphate, the immediate energy source of the reaction.

63 male rats weighing between 280 and 300 gm. were used in this study. They were tested for their ability to acetylate p-aminobenzoic acid (PAB) and then divided at random into two main groups. Twenty-one were used as normal non-diabetic controls, while the remaining forty-two were rendered severely diabetic by the intravenous injection of 40 mg. of alloxan monohydrate per kilo of body weight. From 25 to 35 gm. of sugar were excreted per rat per day, approximately 10 per cent of the body weight, and water intake ranged from 250 to 300 ml. per day, approximately. Subgroups of seven animals of each type were used in the tests described below. These groups were used repeatedly but the acetylating ability of each rat was always determined prior to the administration of any test material to be sure that the animal had returned to the basal level of acetylation. All animals received a purified diet (11) containing sucrose as the carbohydrate and were kept in individual metabolism cages.

Acetylation was tested by injecting PAB intraperitoneally, collecting the following 24 hour urine, and analyzing it for total and free PAB according to the method of Bratton and Marshall (12). Unless otherwise specified, 1 mg. of PAB was injected. The amount acetylated was calculated as the difference between total and free PAB. The values for total PAB excreted are reported as per cent of the dose excreted in the urine during the 24 hour interval.

Results

The rats of the first group and those of the second group, before the latter were made diabetic, acetylated 76 per cent of the test dose of PAB (1 mg.). In the animals rendered diabetic acetylation gradually fell to 62 per cent, as shown in Fig. 1. Acetylation tests were usually made twice a week on the same animal. This fall in the acetylation in the diabetic rats was found to be highly significant statistically ($P = <0.000001$).

The effect of the size of the dose of PAB on the degree of acetylation was studied, since it has been shown (4) that a decrease in the acetylation in thiamine-, riboflavin-, and pantothenic acid-deficient animals is only evident over a limited range of PAB dosage. Groups of seven diabetic and seven non-diabetic rats were injected with doses of PAB ranging from 0.5 to 10.0 mg. per rat. The results are shown in Fig. 2. It will be seen that the difference in acetylation between the diabetic and the non-diabetic rats gradually became smaller as the dose of PAB increased. The difference is significant with doses of PAB ranging from 0.5 to 3.0 mg.

Since the volume of urine is large in the diabetic animals (150 to 230 ml.), and this might hasten the excretion of PAB and lower the extent of acetylation, the effect of diuresis on acetylation in normal rats was studied.
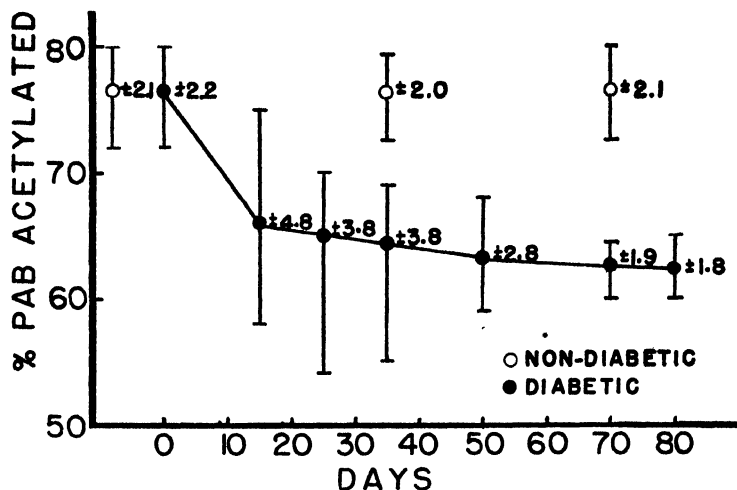
Fig. 1. Changes in acetylation with time after the production of alloxan diabetes. The length of the lines indicates the range of values obtained at each time. The figures are the standard deviation of each group.
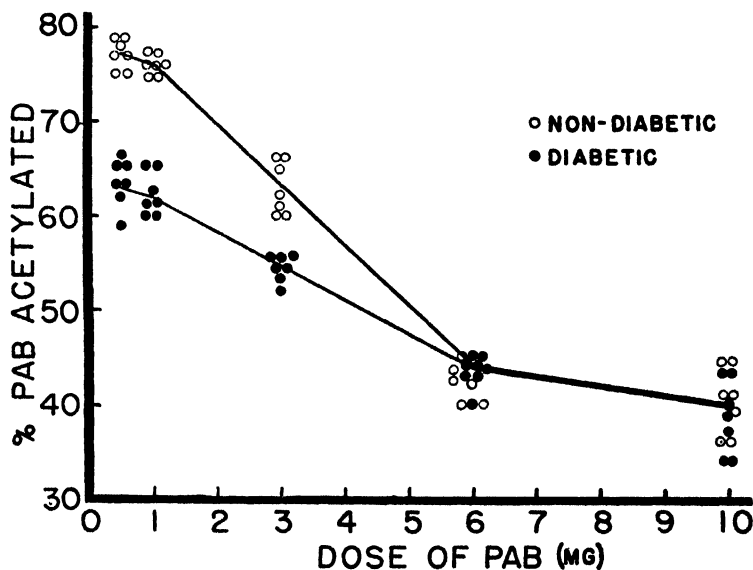
FIG. 2. The relationship of the size of the dose of PAB to the per cent acetylated in normal and diabetic rats.

Food was withheld from eleven non-diabetic rats which were given solutions of 5 per cent glucose or 8 per cent sucrose as their drinking water for

a period of 24 hours, during which time acetylation of PAB was tested. Table I summarizes the data with regard to liquid intake, urinary volume, and per cent PAB acetylated. Although the severity of the induced diuresis was comparable to that of the diabetic rats, it had no effect on acetylation. The diabetic rats and the non-diabetic animals with induced diuresis excreted practically all of the injected PAB within the first 2½ hours.

Pantothenic acid is part of the coenzyme necessary for the acetylation reaction, and, since no information on the requirements of this vitamin by the diabetic rat is available, the possibility that the decreased acetylation in the diabetic animals might be due to increased requirements of this vitamin was investigated. Seven rats were injected with alloxan and as

TABLE I

Effect of Induced Diuresis on Acetylation of PAB in Normal Rats

Liquid intake per 24 hrs. per rat	Carbohydrate concentration		Urinary volume	PAB acetylated
ml.			*ml.*	*per cent*
180	Glucose	5%	135	81
120	"	5%	76	76
50	"	5%	30	79
90	"	5%	60	77
175	Sucrose	8%	140	76
170	"	8%	135	76
170	"	8%	150	77
150	"	8%	125	74
175	"	8%	135	75
150	"	8%	105	77
200	"	8%	155	77

soon as diabetes developed they, together with seven non-diabetic rats, were given the purified diet containing no pantothenic acid. The ability of these animals to acetylate PAB was determined at intervals. When acetylation reached low, constant values, both groups of rats were injected with pantothenic acid and their response compared. As is shown in Fig. 3, the acetylation in the diabetic rats fell to 51 per cent in 30 days, while the non-diabetic animals required 115 days to reach the same level. The injection of various doses of pantothenic acid increased the acetylation of the diabetic rats, but the increase did not reach the predeficiency, non-diabetic level and was not influenced by the size of the pantothenic acid doses which were tried. On the other hand, the response of the non-diabetic, deficient rats reached the predeficiency level and was proportional to the size of the dose. It is apparent that lack of pantothenic

acid is not responsible for the low acetylation in the diabetic rat receiving the purified diet, and that the lower acetylation in the diabetic rat during pantothenic acid deficiency is the result of other factors superimposed on the deficiency.

The possibility that a lack of available acetate in the body resulted in the decreased acetylation was investigated by injecting acetate or other substances that might give rise to acetate. The substances tested were acetate, methyl ester of acetoacetate, pyruvate, lactate, glyceryl diacetate, acetoin, butylene glycol, diacetyl, and acetyl phosphate. All substances were given subcutaneously in isotonic concentrations. With the exception of diacetyl and acetyl phosphate, these materials had no effect on
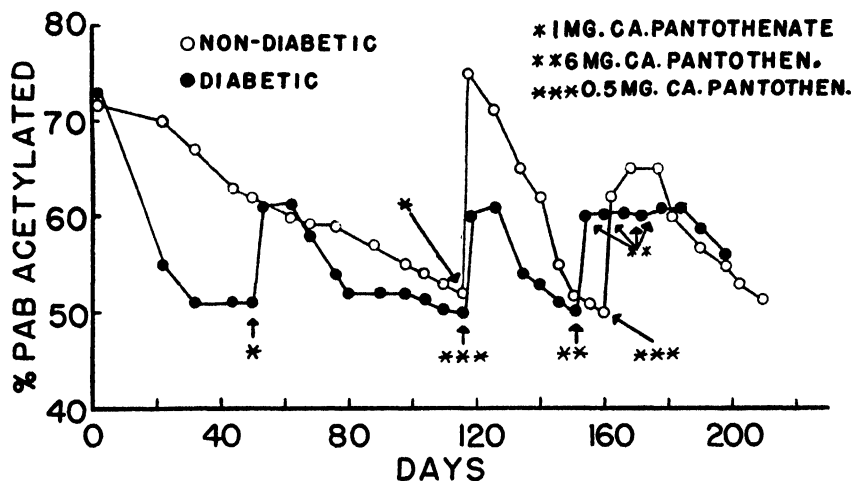
FIG. 3. The effect of pantothenic acid deficiency and calcium pantothenate injections upon the acetylation of PAB in diabetic and non-diabetic rats.

the acetylation of PAB by either the diabetic or non-diabetic animals. The inactive substances were tested at doses ranging from 0.5 to 6 mM per rat, while diacetyl and acetyl phosphate were active at 116 and 232 μM per rat respectively (Table II). The latter two substances were effective only in the diabetic animals. The inactivity of acetate and presumed acetate precursors suggested that lack of acetate was not the primary defect in the diabetic rats. The activity of acetyl phosphate might be explained on the basis of its function as phosphate donor. Kaplan and Lipmann (13, 14) demonstrated phosphorylation of glucose when acetyl phosphate was added to pigeon liver extracts. They also (15) showed a synthesis of pyruvate when acetyl phosphate, acetate, and ADP[1] were

---

[1] ADP = adenosine diphosphate; ATP = adenosine triphosphate.

TABLE II

Relative Activity of Various Compounds Found Effective in Stimulating Acetylation of PAB in Diabetic Rats

Substance injected	No. of animals		Dose per rat	Per cent PAB acetylated			
	Diabetics	Controls		Diabetics		Controls	
				Before	After	Before	After
			μM				
Diacetyl	14		116	64	68*		
"	35	7	232	63	76*	76	75
"		7	1000			76	77
Citrate	7		250	64	65		
"	7		370	62	66*		
"	7	7	500	63	78*	76	75
α-Ketoglutarate	7		100	64	65		
"	7		125	62	72*		
"	7		250	64	76*		
"	7	7	500	62	77*	76	77
Succinate	7		125	63	64		
"	7		170	64	69*		
"	7		250	64	73*		
"	7		500	62	76*		
"	7	7	1000	64	77*	76	77
Fumarate	7		125	63	64		
"	7		170	63	71*		
"	7		250	64	75*		
"	7		500	63	76*		
"	7	7	1000	64	78*	75	77
Malate	7		60	63	63		
"	7		80	64	71*		
"	7		100	64	75*		
"	7		250	63	77*		
"	7		500	64	78*		
"		7	1000			77	79
Oxalacetate	7		60	63	64		
"	7		80	63	65		
"	7		125	65	75*		
"	7		170	63	74*		
"	7	7	250	63	76*	76	76
Acetyl phosphate	14	7	232	63	77*	77	78
ATP	7		15	63	65		
"	7		20	62	69*		
"	7		30	63	74*		
"	7	7	100	63	77*	76	79
"	3		250	63	81*		

* ($P < 0.01$), significant increase.

added to *Escherichia coli* extracts and interpreted the results as due to the formation of ATP[1] from the interaction of acetyl phosphate and ADP.

The reaction product of the resulting ATP and acetate is the acetyl donor for the synthesis of pyruvate. According to the same authors this reaction product of ATP and acetate is not identical with synthetically prepared acetyl or diacetyl phosphate, because these substances do not function as acetyl donors in the *in vitro* system.

In view of the activity of acetyl phosphate in raising acetylation in the diabetic rats and the necessity of ATP as the energy source for the reaction, the effect of injections of ATP and other substances whose oxidation is coupled with phosphorylation through the formation of ATP was investigated. The substances tried were ATP, compounds of the tricarboxylic acid cycle, adenylic acid (both yeast and muscle), and phosphate salts. All were injected subcutaneously. With those substances that were found effective in raising the extent of acetylation, the relation be-
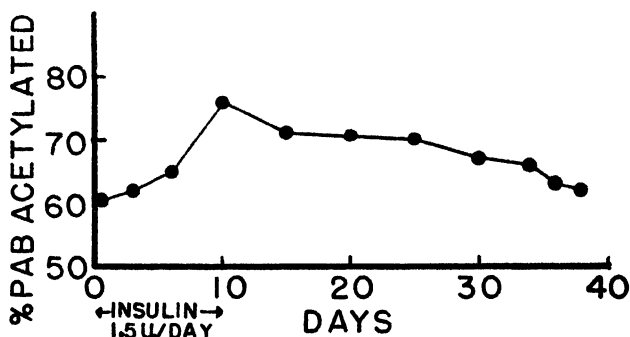
FIG. 4. Effect of protamine zinc insulin injections upon the acetylation of PAB in diabetic rats.

tween dosage and acetylation was also investigated to determine the minimum effective amount.

As is shown in Table II, the members of the tricarboxylic acid cycle tested were effective in increasing the extent of acetylation in diabetic rats to normal levels. Essentially maximum acetylation values were obtained with 80 μM of sodium malate. Oxalacetate and α-ketoglutarate appeared somewhat less active, succinate and fumarate still less active, and citrate the least active of those materials which affected the acetylation. ATP was much more active than any of the other materials studied and produced maximum acetylation at a level of 20 to 30 μM per rat. Adenylic acid and phosphate salts were inactive, thus indicating that the activity of ATP is due to the high energy pyrophosphate bond it contains.

It may be noted that none of the materials active in the diabetic rats had any effect upon the acetylation in the control animals. Also, that

the acetylation in the diabetic rats was never increased above the normal level even at very high dosages of the various substances tested.

It is well known that an increase in the level of fat in the diet will ameliorate the diabetic symptoms in the alloxan-diabetic rat (16). In our animals an increase in the fat content of the diet from the original level of 5 per cent up to 30 per cent raised the acetylation values to normal. 24 hours of fasting produce the same effect as does the administration of insulin. However, the effect of insulin is much delayed. As is shown in Fig. 4, approximately 10 days were required to obtain normal acetylation, although the amount of insulin given (1.5 units of protamine zinc insulin daily) was sufficient to produce an abrupt fall in urinary sugar and urinary volume. Similarly, after the administration of insulin was stopped, the diuresis became maximum within 3 days, but the acetylation values fell gradually and reached the preinsulin level only after 26 days. Acetylation in normal animals was not influenced by daily injections of 0.5 unit of protamine zinc insulin for 10 days.

DISCUSSION

The data show that under the conditions used in these studies the acetylation of PAB in the diabetic rat gradually falls after the onset of diabetes and that this decrease in acetylation is not due to a lack of pantothenic acid or the diabetic diuresis. With regard to the latter factor, it may be noted that not only was induced diuresis in normal animals ineffective in changing the extent of acetylation, but that maximum diuresis after withdrawal of insulin in the diabetic animal is obtained within a few days, while acetylation falls gradually over a long period of time.

The results can be interpreted most readily if one assumes that by the use of small amounts of PAB as the test substance a measure of the overall rate of metabolic acetylations is obtained; that is, that no stress is placed upon any portion of the acetylating system and the amount of PAB acetylated is related to the rate of acetylation in the body at that time. On the other hand, when large doses of PAB or foreign amines are administered, a load may be placed upon the acetylating system and the test probably becomes a measure of the amount of acetyl donors available. It is also possible that under these conditions the normal metabolic processes may be diverted to cover the load placed upon the system. If this is the proper interpretation of the test, it may explain apparent discrepancies between this work and previous studies which showed that acetate, pyruvate, lactate, glycerol (17), butylene glycol, and acetoin (18) would increase acetylation of aromatic amines in normal rabbits. These materials were not effective in either diabetic or normal rats in the present studies at levels much above those used for the members of the tricarboxylic acid cycle. Species differences may also be involved but the

level of foreign amine administered appears to be an important factor.

One might also speculate that, if the test as used was a measure of the extent of the specific reaction, PAB→acetyl-PAB, rather than a measure of the over-all metabolic acetylations, a stimulation of the tricarboxylic acid cycle might be expected actually to draw 2 carbon fragments into the cycle and thus decrease the amount available for the acetylation of PAB.

The common characteristic of all the materials, with the exception of diacetyl, which were found active in raising the acetylation in diabetic rats is that they contain a high energy phosphate bond (ATP and acetyl phosphate) or that they stimulate oxidation which should result in a net gain of utilizable energy in this form (19–25). On the basis of the *in vitro* work previously cited (7–10) and the fact that ATP itself was the most active material tested, it is reasonable to assume that a primary deficiency of ATP is the cause of the metabolic defect and that the activity of other materials resides in their ability to stimulate the formation of ATP. The activity of acetyl phosphate is most likely due to its reaction with ADP to form ATP as mentioned previously.

This explanation is consistent with the demonstration by Kaplan and Greenberg (26) that the ATP content of the livers of rats during diabetic coma is much below normal levels. He also found an increase in ATP after insulin administration (27) and an increase in the rate of incorporation of P^{32} into the molecule.

The activity of diacetyl apparently cannot be explained on the same basis at the present time, and is perhaps surprising in view of the inactivity of butylene glycol and acetoin. It may act as an acetyl donor directly. For the explanation of the effect of high fat diets or fasting in increasing acetylation, one may assume that the energy derived from the breakdown of fat may be made available as ATP without the intervention of insulin.

The action of insulin itself on acetylation in doses sufficiently large to cure the obvious signs of diabetes is very slow in comparison with that of the other materials tested. After the withdrawal of insulin, the glycosuria and urine volume were maximum after 3 days, but acetylation reached a minimum value only after 3 weeks. Thus, as measured by the acetylation test, insulin exerts an influence for a much longer time than one would assume from other measures of the degree of diabetes. Although the mechanism of this is not clear, it is possible that some of the difficulties in obtaining consistent results in studies on the site of action of insulin *in vitro* and *in vivo*, and discrepancies in the literature, may be related to this prolonged insulin effect. Kaplan *et al.* (25) also found that extremely high doses of insulin were required to raise the ATP level in the livers of diabetic rats.

It is of some interest that pyruvate was inactive in these tests, although

most schemes for the metabolism of this material indicate that it should be decarboxylated and enter the tricarboxylic acid cycle by condensing with oxalacetic acid without the intervention of insulin. Recent work indicates that insulin functions on the hexokinase reaction either indirectly, by releasing the inhibitory action of the anterior pituitary (28), or directly by accelerating the formation of glucose-6-phosphate from glucose (29), or both. Thus a deficiency of insulin would be expected to decrease the available pyruvate and this, in turn, would slow down the rate of turnover of the tricarboxylic acid cycle. However, if this were the only defect in diabetes, one would anticipate a stimulation of the tricarboxylic acid cycle by an increase in pyruvate supply. The inability of large amounts of pyruvate or lactate to increase acetylation in these studies indicates that pyruvate alone cannot stimulate the formation of the acids of the tricarboxylic acid cycle in the diabetic animal. The data, therefore, suggest the possibility that insulin may be involved in metabolic reactions upon which the utilization of pyruvate depends, presumably between pyruvate and the tricarboxylic acid cycle. The dicarboxylic acids have been shown (30, 31) to be required for pyruvate oxidation.

Recent studies by Stadie and Haugaard (32) have failed to confirm the hexokinase reaction as the site of action of insulin. The diabetes in their animals was very mild and most of the work was done 48 hours after alloxan injection. There is, however, considerable scattered evidence that pyruvate utilization may be directly influenced by insulin. A careful examination of the data on pyruvate tolerance in diabetic dogs by Flock et al. (33) shows that the one dog which had been without insulin for 3 days had a high and much prolonged blood pyruvate level after pyruvate administration. Blood lactate also reached very high levels. Similar but less spectacular results were observed in the second diabetic dog which had been without insulin for 48 hours. It seems probable, on the basis of the present studies, that these animals may still have been under the influence of insulin in spite of the high blood sugar levels. Bloch and Kramer (34) found that the rate of incorporation of C^{14} into fatty acids by liver slices was increased by insulin when pyruvate, but not glucose, was the substrate. Rice and Evans (35) observed increased oxygen consumption and pyruvate utilization when insulin was added to pigeon muscle mince. The insulin effect was inhibited by malonate and this could be reversed by fumarate or oxalacetate. Krebs and Eggleston (36) and others (37) observed an increase of the respiration of pigeon breast muscle mince after the addition of insulin and suggested that insulin may function in some step in the tricarboxylic acid cycle. This insulin effect was not observed by Shorr and Barker (38) when muscle mince from other animals than pigeons, e.g. cat, dog, and rabbit, was used. Similarly Stare and Baumann (39) failed

to observe a marked increase of respiration under the experimental conditions originally outlined by Krebs and Eggleston, but, when a Ringerphosphate solution containing 0.2 per cent glucose was used, insulin increased respiration, particularly in the breast muscle of depancreatized pigeons (40).

The possibility that members of the tricarboxylic acid cycle might exert a beneficial effect in human diabetes has been investigated by various workers. Succinic acid was reported to decrease ketonuria in human diabetes (41, 42), to have no effect (43, 44), or actually to increase ketonuria (45). Inconsistent results were also obtained with citric acid when given to diabetic patients (46), to phlorhizinized dogs (47), and to rats on ketogenic diets (48).

SUMMARY

1. Acetylation of p-aminobenzoic acid in diabetic rats was found to be significantly below the level of that in non-diabetic controls.

2. Acetylation returned to the normal level after the injection of insulin, dicarboxylic acids of the tricarboxylic acid cycle, adenosine triphosphate, acetyl phosphate, and diacetyl. Fasting or feeding a high fat diet had a similar effect.

3. Acetate, acetylmethyl acetate, pyruvate, lactate, glyceryl diacetate, acetoin, butylene glycol, phosphate salts, adenylic acid, and pantothenic acid failed to increase acetylation.

4. None of the compounds tested increased acetylation in the non-diabetic rats.

5. The influence of insulin on oxidative phosphorylations and acetylations is discussed. The data suggest a function of insulin in the utilization of pyruvate.

BIBLIOGRAPHY

1. Lipmann, F., Kaplan, N. O., Novelli, G. D., Tuttle, L. C., and Guirard, B. M., *J. Biol. Chem.*, **167**, 869 (1947).
2. Riggs, T. R., and Hegsted, D. M., *J. Biol. Chem.*, **172**, 539 (1948).
3. Olson, R. E., and Kaplan, N. O., *J. Biol. Chem.*, **175**, 515 (1948).
4. Riggs, T. R., and Hegsted, D. M., *J. Biol. Chem.*, **178**, 669 (1949).
5. Klein, J. R., and Harris, J. S., *J. Biol. Chem.*, **124**, 613 (1938).

6. Lipmann, F., *Advances in Enzymol.*, **1**, 99 (1941).
7. Lipmann, F., *J. Biol. Chem.*, **160**, 173 (1945).
8. Nachmansohn, D., and Machado, A. L., *J. Neurophysiol.*, **6**, 397 (1943).
9. Mann, P. J. G., Tennenbaum, M., and Quastel, J. H., *Biochem. J.*, **32**, 245 (1938).
10. Lipmann, F., *Advances in Enzymol.*, **6**, 231 (1946).
11. Lowry, P. T., and Hegsted, D. M., *J. Lab. and Clin. Med.*, **30**, 839 (1945).
12. Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, **128**, 537 (1939).
13. Kaplan, N. O., and Lipmann, F., *Federation Proc.*, **7**, 163 (1948).
14. Lipmann, F., *J. Biol. Chem.*, **155**, 55 (1944).
15. Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **176**, 459 (1948).
16. Burn, J. H., Lewis, T. H. C., and Kelsey, F. D., *Brit. Med. J.*, **2**, 752 (1944).
17. Harrow, B., Mazur, A., Borek, E., and Sherwin, C. P., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **105**, p. xxxiv (1934).
18. Doisy, E. A., Jr., and Westerfeld, W. W., *J. Biol. Chem.*, **149**, 229 (1943).
19. Colowick, S. P., Welch, M. S., and Cori, C. F., *J. Biol. Chem.*, **133**, 359 (1940).
20. Kalckar, H., *Enzymologia*, **6**, 209 (1939).
21. Colowick, S. P., Kalckar, H. M., and Cori, C. F., *J. Biol. Chem.*, **137**, 343 (1941).
22. Kalckar, H. M., *Biochem. J.*, **33**, 631 (1939).
23. Belitzer, V. A., and Tsybakova, E. T., *Biokhimiya*, **4**, 516 (1939).
24. Ochoa, S., *Nature*, **146**, 267 (1940).
25. Kaplan, N. O., Franks, M., and Friedgood, C. E., *Science*, **102**, 447 (1945)
26. Kaplan, N. O., and Greenberg, D. M., *J. Biol. Chem.*, **156**, 553 (1944).
27. Kaplan, N. O., and Greenberg, D. M., *J. Biol. Chem.*, **150**, 479 (1943).
28. Cori, C. F., *Harvey Lectures*, **41**, 253 (1945).
29. Perlmutter, M., and Greep, R. O., *J. Biol. Chem.*, **174**, 915 (1948).
30. Banga, I., Ochoa, S., and Peters, R. A., *Biochem. J.*, **33**, 1980 (1939).
31. Ochoa, S., *J. Biol. Chem.*, **138**, 751 (1941).
32. Stadie, W. C., and Haugaard, N., *J. Biol. Chem.*, **177**, 311 (1949).
33. Flock, E., Bollman, J. L., and Mann, F. C., *J. Biol. Chem.*, **125**, 49 (1938).
34. Bloch, K., and Kramer, W., *J. Biol. Chem.*, **173**, 811 (1948).
35. Rice, L., and Evans, E. A., *Science*, **97**, 470 (1943).
36. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **32**, 913 (1938).
37. Stadie, W. C., Zapp, J. A., Jr., and Lukens, F. D. W., *J. Biol. Chem.*, **132**, 411 (1940).
38. Shorr, E., and Barker, S. B., *Am. J. Physiol.*, **126**, 629 (1939).
39. Stare, F. J., and Baumann, C. A., *J. Biol. Chem.*, **133**, 453 (1940).
40. Stare, F. J., and Baumann, C. A., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 277 (1939).
41. Korányi, A., and Szent-Györgyi, A., *Deut. med. Wochschr.*, **63**, 1029 (1937).
42. Brockmüller, A., *Münch. med. Wochschr.*, **85**, 252 (1938).
43. Lawrence, R. D., McCance, R. A., and Archer, N., *Brit. Med. J.*, **2**, 214 (1937).
44. MacKay, E. M., Sherill, J. W., and Barnes, R. H., *J. Clin. Invest.*, **18**, 301 (1939).
45. Dibold, H., Frey, L., and Lapp, F. W., *Deut. med. Wochschr.*, **63**, 1505 (1937).
46. Connavo, L., and Ortoleva, G., *Riforma méd.*, **56**, 1437 (1940).
47. Fürth, O., Minnibeck, H., and Edel, E., *Biochem. Z.*, **269**, 379 (1934).
48. MacKay, E. M., Carne, H. O., and Wick, A. N., *J. Biol. Chem.*, **133**, 59 (1940).

THE UTILIZATION OF AMINO ACIDS AND PEPTIDES BY MUTANT STRAINS OF ESCHERICHIA COLI*

BY SOFIA SIMMONDS AND JOSEPH S. FRUTON

(From the Osborn Botanical Laboratory and the Department of Physiological Chemistry, Yale University, New Haven)

In recent communications (1–3), data have been presented concerning the growth-promoting effect of peptides on artificially produced mutant strains of *Escherichia coli* which require certain amino acids for growth. In these studies, the extent of bacterial growth in a given time was plotted against the concentration, in the medium, of the appropriate amino acid or of related peptides. In this manner, it was possible to estimate the concentration of each growth factor necessary for the production of half maximal growth. Thus, it was found that equimolar concentrations of L-phenylalanine and of glycyl-L-phenylalanine were required by a *phenylalanineless* mutant for the promotion of half maximal growth in 24 hours (1). In the case of a *prolineless* mutant, however, the molar concentration of glycyl-L-proline which gave half maximal growth in 48 hours was only one-half the concentration of L-proline required to produce the same effect (3). With a *leucineless* mutant it was observed that, at low concentrations, equimolar amounts of L-leucine and of glycyl-L-leucine promoted growth in 24 hours to the same extent. At concentrations above that which caused maximal growth, however, the dipeptide exerted an inhibitory action on the growth of the mutant strain (2).

It was evident, therefore, that, although these three mutant strains of *Escherichia coli* can utilize, for growth, peptides derived from the appropriate amino acids, the mutants exhibited striking differences in their response to peptides. In view of these differences, it appeared desirable to examine the growth response of the mutants to amino acids and peptides as a function of time as well as of the concentration of the test substances.

In the studies described in the present communication, the synthetic minimal medium, consisting of inorganic salts, glucose, and a small amount of asparagine (4), was supplemented with the appropriate amino acid or peptide and dispensed into Evelyn colorimeter tubes in 10 ml. amounts. In what follows, the concentration of test compounds is expressed as μM per 10 ml. of medium. The pH of the media was adjusted to 7.1 to 7.2 with N NaOH as required.

The three bacterial strains used were double mutants of the wild type strain K-12 (5). The *phenylalanineless* strain (No. 58-278) required biotin as well as L-phenylalanine. The minimal medium for this mutant, therefore, always contained sufficient biotin to satisfy the requirement for this compound (1). The *prolineless* and the *leucineless* strains (Nos. 679-183 and 679-680, respectively) exhibited an additional requirement for L-threonine. Since these studies were concerned only with the proline and leucine requirements, the minimal medium for these strains was supplemented with enough DL-threonine to meet the requirement for this amino acid (2, 3).

After sterilization in the autoclave, the tubes were inoculated with cells harvested from a peptone-yeast extract-agar slant that had been incubated for 24 hours at 30° after inoculation. To provide uniform inocula for all tests, an aqueous suspension of cells was prepared with an optical density of 0.028 to 0.032, equivalent to a galvanometer reading in the Evelyn colorimeter (filter No. 540) of 93.00 to 93.75. In several experiments, cells grown in liquid medium were used for the inocula. In these cases, a cell suspension having the optical density given above was prepared by dilution of the culture with the required amount of sterile water. In all experiments, 0.1 ml. of the bacterial suspension was added to each tube and the tubes were incubated at 30°. The extent of bacterial growth was measured turbidimetrically, in the Evelyn colorimeter, at appropriate intervals. During the logarithmic phase of growth, readings were taken every 90 to 120 minutes. To facilitate inspection of the growth curves given in Figs. 1 to 6, the observed density readings have been omitted.

Phenylalanineless Strain—The response of the *phenylalanineless* strain to several concentrations (0.046 to 1.8 μM) of L-phenylalanine and of glycyl-L-phenylalanine was followed by the method described above. For each concentration of test substance, the density of the bacterial culture was plotted as a function of time, and it was found that, at equimolar concentrations, the growth curves for the amino acid and the dipeptide were identical (Fig. 1). An increase in the concentration of L-phenylalanine or of glycyl-L-phenylalanine above that which promoted maximal growth (approximately 0.3 μM) caused no change in the growth curve. At concentrations below 0.3 μM, the height of the growth curve depends upon the concentration, in the medium, of phenylalanine, either as the free amino acid or as the dipeptide. It may be concluded, therefore, that phenylalanine supplied to the organism in the form of glycyl-L-phenylalanine is utilized for growth as effectively as is L-phenylalanine. If the utilization of the dipeptide for growth depends upon its hydrolysis to glycine and phenylalanine prior to the utilization of the phenylalanine which is liberated, this hydrolysis must proceed at an extremely rapid rate and

cannot be the limiting factor in the initiation of growth or in the rate of growth of the mutant.

Prolineless Strain—Earlier experiments showed that glycyl-L-proline promoted the growth of the *prolineless* mutant more effectively than did an equivalent concentration of L-proline (3). In view of the possibility that the L-proline used in these studies contained traces of an impurity which acted as a growth inhibitor, the growth-promoting activity of the free amino acid was compared with that of an acid hydrolysate of the peptide. 30 mg. samples of L-proline and of glycyl-L-proline were boiled with 1 ml. of 20 per cent hydrochloric acid under a reflux for 6 hours. To each
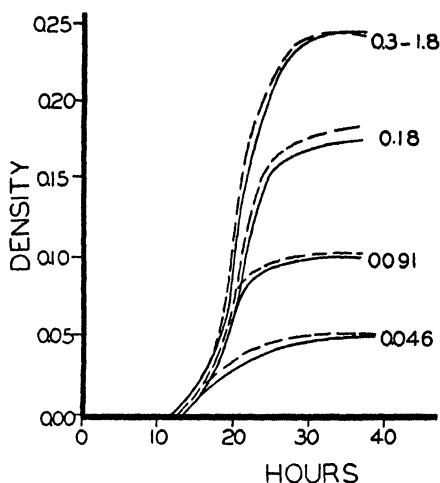
Fig. 1. Representative growth curves for the *phenylalanineless* mutant in the presence of L-phenylalanine (solid lines) and of glycyl-L-phenylalanine (dash lines). The figures to the right of the curves denote the concentrations of the test compounds in μM per 10 ml.

acid solution about 30 ml. of water were added, and the excess HCl was removed by concentration to dryness *in vacuo*. The residue was taken up in a small amount of water, and the solution was neutralized with N NaOH. The resultant solutions of acid-treated L-proline and of hydrolyzed glycyl-L-proline, as well as an untreated sample of L-proline, were assayed for their growth-promoting activity for the *prolineless* mutant. At the concentrations of proline used (0.1 to 10 μM), the three solutions showed identical activities. The L-proline preparation used in this and earlier studies does not appear, therefore, to contain an inhibitor of the growth of the *prolineless* mutant.

Confirmation of the earlier observations on the relative activities of

L-proline and glycyl-L-proline was obtained when the growth of the *prolineless* mutant was studied as a function of time. As is shown in Fig. 2, at low concentrations (0.12 to 0.44 μM) the dipeptide was about twice as effective as an equimolar concentration of the free amino acid, and the optical density ultimately obtained with any given concentration of L-proline was never as great as that obtained with an equimolar concentration of glycyl-L-proline.

At least three possibilities may be considered in connection with this phenomenon. In the first place, the peptide may be incorporated into the bacterial proteins as such, without prior hydrolysis. While this cannot be ruled out completely, it is made less attractive by the fact, reported
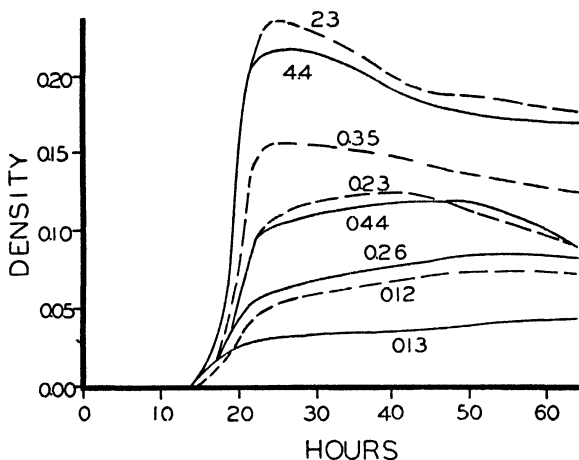
Fig. 2. Representative growth curves for the *prolineless* mutant in the presence of L-proline (solid lines) and of glycyl-L-proline (dash lines). The concentration of the test compound, in μM per 10 ml., is indicated for each curve.

earlier (3), that all proline peptides tested with this *prolineless* mutant were more effective in promoting bacterial growth than was the parent amino acid. A second possibility is that a part of the proline supplied to the organism as the free amino acid is converted, in the course of bacterial metabolism, to products which are not utilized for growth, while the proline supplied in the form of peptides is less readily converted to such products. This explanation finds an analogy in the report of Gale (6) concerning the greater growth-promoting activity for Group D streptococci of acid-treated salmine as compared with arginine. In addition to these two possibilities, it must be considered that the presence of proline in a peptide linkage may make the amino acid more readily available for protein synthesis, perhaps by a mechanism involving "transpeptidation" reactions. It is hoped that further work with the *prolineless* mu-

tant will indicate whether any of these possibilities is operative in the peptide metabolism of this organism.

Leucineless Strain—It had previously been found (2) that, while glycyl-L-leucine and L-leucine were equally effective, on a molar basis, with respect to the production of half maximal growth of the *leucineless* mutant in 24 hours, the dipeptide was markedly inhibitory at concentrations above 1.5 μM. At these high concentrations, neither L-leucine nor a mixture of L-leucine and glycine inhibited growth. It was also found that, in high concentrations, the dipeptide was inhibitory to the growth of the wild type strain (K-12) which can synthesize leucine from the constituents of the minimal medium.

Preliminary to the further investigation of the inhibitory action of glycyl-L-leucine, an examination was made of the growth-promoting activity of an acid-hydrolyzed sample of the peptide. Samples of the peptide, of L-leucine and of an equimolar mixture of L-leucine and glycine were treated with 20 per cent hydrochloric acid in the manner described above for the hydrolysis of glycyl-L-proline. The growth-promoting activity of the final solutions of hydrolyzed glycyl-L-leucine, of L-leucine, and of L-leucine plus glycine was then determined by using the *leucineless* mutant. As controls, a sample of untreated L-leucine and a mixture of untreated L-leucine and glycine were also assayed at the same time. The concentrations of the test substances ranged from 0.1 to 4.8 μM, and the extent of growth was observed at 15, 17.5, 24, and 48 hours after inoculation. At all times the same growth response was obtained with equivalent concentrations of all five test solutions. Thus, the inhibitory action of glycyl-L-leucine appears to be a property of the dipeptide *per se*.

The relative growth-promoting properties of L-leucine and of glycyl-L-leucine were then studied as a function of time, at concentrations of the test compounds ranging from 0.1 to 4.8 μM (Fig. 3). It will be seen from the curves in Fig. 3 that, in contrast to the results with the *phenylalanine-less* and *prolineless* mutants, the duration of the lag phase of the *leucineless* mutant was greater in the presence of the peptide than of the amino acid, and that the initiation of growth in the presence of increasing concentrations of the peptide required increasingly longer time intervals. Thus, if the activities of L-leucine and of glycyl-L-leucine were compared only after 24 hours, the higher concentrations of the dipeptide would appear to exert an inhibitory effect on growth. However, the extent of bacterial growth that was ultimately attained in media containing large amounts of glycyl-L-leucine was essentially the same as that observed in the presence of corresponding amounts of L-leucine. In these experiments, therefore, glycyl-L-leucine exerted a significant inhibitory effect only upon the initiation of rapid growth.

It seemed possible that the growth of the *leucineless* mutant in the presence of glycyl-L-leucine might depend upon the prior development of adaptive enzymes necessary for the conversion of the dipeptide to a compound more readily utilized for growth. To test this possibility, the response of the mutant to L-leucine and to glycyl-L-leucine was compared when, in one case, the inoculum consisted of cells grown in minimal medium supplemented with L-leucine (2.4 μM) and, in a second case, of cells grown in minimal medium supplemented with glycyl-L-leucine (2.4 μM). In both cases, the inocula were harvested at a time corresponding to the end of the logarithmic phase of growth. No difference was observed in the growth response of the two inocula when these were tested in the presence of
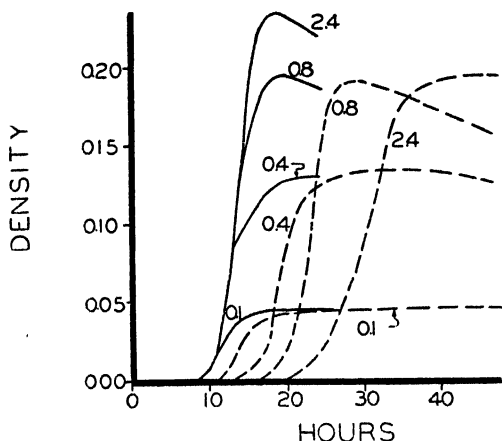
FIG. 3. Representative growth curves for the *leucineless* mutant in the presence of L-leucine (solid lines) and of glycyl-L-leucine (dash lines). The inoculum was taken from a peptone-yeast extract-agar slant incubated for 24 hours. The concentration of the test compound, in μM per 10 ml., is indicated for each curve.

glycyl-L-leucine. Also, the two inocula gave the same response with L-leucine. The behavior of the inocula obtained from the peptide-containing medium was indistinguishable, therefore, from that of the inocula obtained from the medium containing L-leucine. A second set of transfers to fresh media of cells grown on L-leucine or on glycyl-L-leucine still did not show any difference in the growth response of the two inocula in the presence of the peptide. In view of these results, it appears unlikely that the growth of the *leucineless* mutant in the presence of glycyl-L-leucine involves the production of enzymes that are required for the utilization of the peptide for growth, and which are not also produced by cells grown in the presence of L-leucine.

In the course of the experiments designed to study the possible forma-

tion of adaptive enzymes, it was found that, with inocula consisting of actively multiplying cells, the difference in the growth response toward glycyl-L-leucine and L-leucine was less marked than in the case of inocula from agar slants. Comparison of the curves in Figs. 3 and 4 shows that, as was to be expected, the "young" inocula exhibited a shorter lag period than did the "older" inocula, and that this effect is especially striking with respect to the growth response to glycyl-L-leucine. Thus the cells in the "young" inocula were able to use the peptide more effectively for
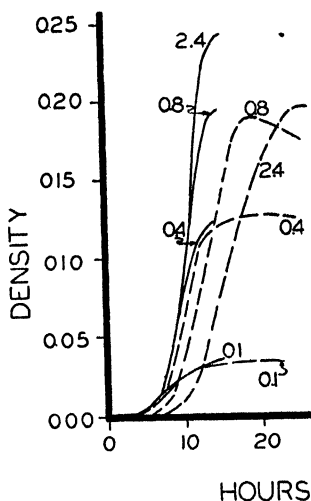
FIG. 4. Representative growth curves for the *leucineless* mutant when "young" cells are used as the inoculum. The inoculum consisted of cells grown in the presence of 2.4 μM of glycyl-L-leucine or of L-leucine, and was harvested 2 hours before the attainment of maximal growth. Solid lines, growth in the presence of L-leucine; dash lines, growth in the presence of glycyl-L-leucine. The concentration of the test compound, in μM per 10 ml., is indicated for each curve.

growth because the increasingly longer lag periods caused by increasing concentrations of the dipeptide were now much less evident.

A second transfer to fresh media of actively multiplying cells cultured in the · presence of L-leucine or of glycyl-L-leucine resulted in no further changes in the duration of the lag phase. On the other hand, when cells were allowed to "age" in minimal medium containing L-leucine or glycyl-L-leucine for 85 to 95 hours after maximal growth had been attained, transfer to fresh media gave growth responses with even more pronounced lag periods than those shown in Fig. 3. Thus, the lag phase in the presence of L-leucine was about 10 hours longer than it had been with an inoculum from the agar slant, and the initiation of growth in the presence of glycyl-

L-leucine required 13 to 18 hours longer than it had before, depending on the concentration of the peptide.

Previous experiments had shown that the addition of high concentrations of glycyl-L-leucine to a medium containing sufficient L-leucine to permit half maximal growth of the *leucineless* mutant in 24 hours did not spare the requirement for L-leucine (2); the extent of growth was no greater than that obtained with the same amount of L-leucine in the absence of glycyl-L-leucine. In view of the above results which showed that the peptide acts as an inhibitor of the initiation of growth of the *leucineless* mutant, it appeared desirable to examine more closely the effect of the peptide on
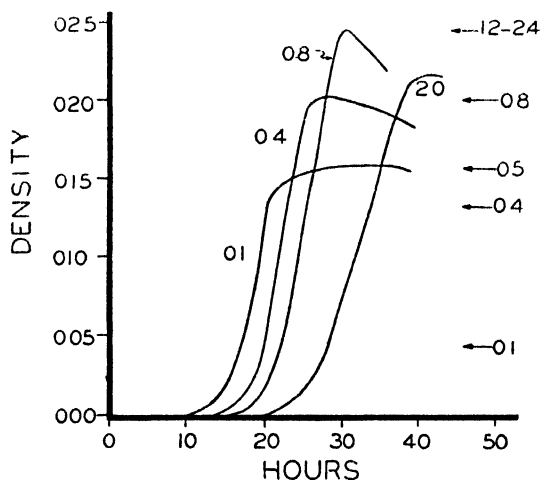
Fig. 5. Representative growth curves for the *leucineless* mutant in media containing 0.4 μM of L-leucine and varying amounts of glycyl-L-leucine. The concentration of the dipeptide, in μM per 10 ml., is indicated for each curve. The arrows at the right of the figure denote the maxima of growth curves obtained with media containing the indicated concentrations of L-leucine in the absence of the dipeptide.

the growth of the mutant in the presence of L-leucine. Mutant cells, taken from agar slants, were inoculated into media containing a constant amount of L-leucine (0.4 μM) and varying amounts of glycyl-L-leucine (0.1 to 2.4 μM). As will be seen in Fig. 5, for each dipeptide concentration, the duration of the lag phase was the same as that previously found with glycly-L-leucine in the absence of L-leucine. The maximum of each curve, however, depended upon the total concentration of L-leucine, both as such and in the form of the dipeptide. It is of interest that, during the logarithmic phase of growth, no breaks in the curves are evident. In a second experiment, in which media containing a constant amount of glycyl-L-leucine (0.4 μM) and increasing amounts of L-leucine (0.1 to 2.4 μM) were

used, the duration of the lag phase was the same as that noted in the presence of 0.4 μM of glycyl-L-leucine without added L-leucine, and the position of the maxima was determined by the total concentration of L-leucine, as amino acid and peptide.

The results presented above indicate that glycyl-L-leucine exerts an inhibitory effect on the initiation of rapid growth of the *leucineless* mutant, and that this inhibition is not overcome by the presence of L-leucine in the medium. The extent of the inhibition depends on the concentration of the inhibitor and also upon the "age" of the inoculum. A reasonable explanation of this finding is that glycyl-L-leucine inhibits one or more of the enzyme systems involved in the utilization of L-leucine for growth. As a result, rapid growth could not begin until the concentration of the dipeptide had been reduced, possibly by enzymatic hydrolysis, to a level that is no longer inhibitory. This interpretation would serve to explain the progressive increase in the duration of the lag period with increasing concentrations of glycyl-L-leucine. The decreased inhibitory effect of the dipeptide observed with inocula taken from actively multiplying cultures may be due to a higher enzymatic activity of such cultures in the utilization of L-leucine for growth or in the hydrolysis of glycyl-L-leucine.

When glycyl-L-leucine is the sole source of the essential amino acid in the medium, the utilization of the dipeptide for bacterial growth may be assumed to involve prior enzymatic hydrolysis to give free L-leucine. While no definite statement can be made concerning the rate of this reaction, it seems probable that it can proceed fairly rapidly.[1] The results presented above suggest that the utilization of the L-leucine liberated from the dipeptide is inhibited by the unhydrolyzed glycyl-L-leucine, since the duration of the lag phase is dependent upon the initial concentration of the dipeptide, both in the absence and in the presence of free L-leucine in the medium.

It is of interest that, in the presence of sufficient L-leucine to permit bacterial growth, the addition of 10.6 μM of glycyl-D-leucine did not cause a measurable inhibition of growth in 24 hours (2). It would appear, therefore, that the prolongation of the lag phase by glycyl-L-leucine depends on the presence of the L-amino acid residue in the peptide.

In view of the finding that glycyl-L-leucine, at high concentrations, inhibited the initiation of rapid growth of the *leucineless* mutant, it appeared desirable to determine whether other derivatives of L-leucine would show a similar effect. L-Leucinamide acetate was selected for such studies, since it had been found previously (2) that this compound exhibited only about

---

[1] It has been found that aqueous extracts of strain K-12 of *E. coli*, as well as of several mutant strains of this organism, exhibit strong peptidase activity toward glycyl-L-leucine (J. E. Ziegler and J. S. Fruton, unpublished experiments).

7 per cent of the growth-promoting activity of L-leucine, as judged by the relative concentrations required for the production of half maximal growth in 24 hours.

As shown in Fig. 6, the duration of the lag phase in the presence of sufficient L-leucinamide acetate to produce visible growth is similar to that noted in the presence of L-leucine (Fig. 3). However, growth in the presence of the amide was quite slow, except at very high concentrations. For the purpose of comparison, the maxima of the curves obtained with varyint concentrations of L-leucine are indicated at the right of the figure. It
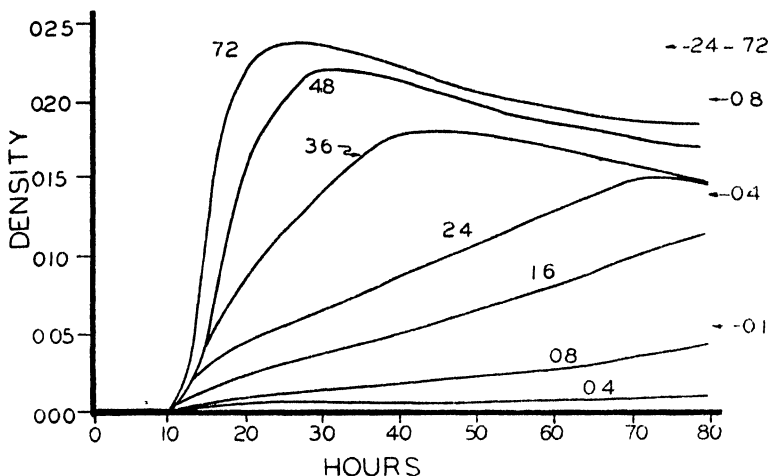
FIG. 6. Representative growth curves for the *leucineless* mutant in the presence of L-leucinamide acetate. The inoculum was taken from a peptone-yeast extract-agar slant incubated for 24 hours, as in Fig. 3. The concentration of the test compound, in μM per 10 ml., is indicated for each curve. The maximal growth in the presence of various concentrations of L-leucine is indicated by arrows at the right of the figure.

is clear, therefore, that the low growth-promoting activity of L-leucinamide acetate, found earlier in short term experiments, was not due to a prolongation of the lag phase, but to a less effective utilization of the amide as a source of L-leucine.

When "young" cells, grown in the presence of L-leucine or of L-leucinamide acetate, were inoculated into fresh media containing these compounds, there was no change in the relative growth-promoting activity of the amino acid and the amide. The duration of the lag phase was shortened to the same extent, but the slopes of the curves and the maxima attained were not altered significantly. It may be added that, when L-leucinamide acetate was subjected to acid hydrolysis, the resulting solu-

tion exhibited the growth-promoting activity to be expected on the basis of the L-leucine formed. These data offer evidence in support of the view expressed earlier (2) that L-leucinamide acetate is probably cleaved by the mutant prior to utilization for growth and that the rate of this cleavage by the bacterial peptidases is relatively slow.

DISCUSSION

The results of the present study point to the fact that, in studies of the bacterial utilization of metabolites, measurement of growth at a single time interval may occasionally be somewhat misleading. The findings with the *leucineless* mutant of *Escherichia coli* emphasize the desirability of determining the complete growth curve since, if peptides such as glycyl-L-leucine are present in the medium, the growth response may be greatly delayed. This has obvious implications in the use of microorganisms for the amino acid assay of complex mixtures such as biological fluids.

It would be premature to attempt to evaluate the general significance of the prolongation of the lag phase by glycyl-L-leucine; further studies, it is hoped, will clarify the possible relationship of this finding to the bacteriostatic action of naturally occurring peptides such as gramicidin. It is clear, however, from the data on the *phenylalanineless* and *prolineless* mutants, that glycyl-L-phenylalanine and glycyl-L-proline, respectively, do not induce the effect observed with the leucine peptide.

The results with the *phenylalanineless* and *leucineless* mutants support the view suggested earlier (1, 2) that, in most cases studied thus far, the utilization of peptides for growth involves prior hydrolysis by the bacterial peptidases, which make the essential amino acid available to the organism. The behavior of the *prolineless* mutant indicates, however, that this may not be the sole metabolic pathway of peptides. In this connection, it may be added that a microorganism has recently been isolated which can utilize L-leucylglycine for growth, but which will not grow in a mixture of the component amino acids (7). It may be expected that the continued study of bacterial strains which exhibit such unusual growth requirements will provide useful data on the metabolic transformations of peptides.

SUMMARY

A study has been made of the growth-promoting activity of amino acids and dipeptides for three mutant strains of *Escherichia coli*. Growth curves were obtained for a *phenylalanineless*, a *prolineless*, and a *leucineless* strain by measuring the extent of bacterial growth as a function of time at varying concentrations of the appropriate amino acids and related peptides.

The *phenylalanineless* strain was found to give exactly the same growth

response to equimolar concentrations of L-phenylalanine and glycyl-L-phenylalanine.

The *prolineless* strain was found to grow about twice as well in the presence of glycyl-L-proline as in the presence of L-proline when growth was limited by the amount of proline in the medium, either as the free amino acid or as the dipeptide. The growth curve for any given concentration of glycyl-L-proline was similar in all respects to the curve for approximately twice the same concentration of L-proline.

With the *leucineless* strain, it was found that the initiation of rapid growth required a longer period in the presence of glycyl-L-leucine than in the presence of L-leucine and that the duration of the lag phase increased with increasing concentrations of the dipeptide in the medium. However, approximately the same amount of bacterial growth was ultimately attained in the presence of equimolar concentrations of L-leucine and glycyl-L leucine. The inhibition of the initiation of growth by the dipeptide was much less evident when the inoculum consisted of cells taken from an actively multiplying culture. The relative response of the mutant to L-leucine and glycyl-L-leucine was found to be independent of the composition of the medium in which the inoculum was cultured.

Growth of the *leucineless* strain in the presence of L-leucinamide acetate was found to be slow, except when the medium contained high concentrations of this compound.

BIBLIOGRAPHY

1. Simmonds, S., Tatum, E. L., and Fruton, J. S., *J. Biol. Chem.*, **169**, 91 (1947).
2. Simmonds, S., Tatum, E. L., and Fruton, J. S., *J. Biol. Chem.*, **170**, 483 (1947).
3. Simmonds, S., and Fruton, J. S., *J. Biol. Chem.*, **174**, 705 (1948).
4. Gray, C. H., and Tatum, E. L., *Proc. Nat. Acad. Sc.*, **30**, 404 (1944).
5. Tatum, E. L., *Proc. Nat. Acad. Sc.*, **31**, 215 (1945).
6. Gale, E. F., *Brit. J. Exp. Path.*, **26**, 225 (1945).
7. Simmonds, S., and Fruton, J. S., *Science*, **109**, 561 (1949).

THE MULTIPLE NATURE OF THE ANIMAL PROTEIN FACTOR

By E. L. R. STOKSTAD, THOMAS H. JUKES, J. PIERCE, A. C. PAGE, Jr.,
AND A. L. FRANKLIN

(From the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York)

Various studies in poultry nutrition have indicated that certain diets based on vegetable products are deficient in a hatchability-promoting or growth-promoting factor which is associated with certain animal protein materials (1–4). Hammond and Titus (5) studied the effect of a diet of corn, soy bean meal, alfalfa meal, butyl fermentation residues, minerals, and vitamin D_3 on chicks and found that growth was improved by adding 2 per cent of sardine meal. The term "animal protein factor" has been used for the dietary factor which is needed for the growth of chicks on diets consisting principally of corn and soy bean meal (6–8). Later work with the diet of Hammond and Titus showed that dried cow manure had an effect similar to that of sardine meal in promoting growth (9).

The studies by Hammond *et al.* were continued by Bird and coworkers who noted that the dietary deficiency was accentuated by increasing the dietary level of soy bean meal to 70 per cent (10). It was also found that the storage of the protective factor in newly hatched chicks was markedly reduced by limiting the supply of the factor in the diet of the parent hens (11, 12). Recently, it was shown by Ott and coworkers (13) that "vitamin B_{12}" was effective in promoting the growth of chicks on a diet which was deficient in the "animal protein factor." This finding was confirmed by Lillie and coworkers (14), who concluded that crystalline vitamin B_{12} was completely effective when tested with chicks and a corn-soy bean meal diet as described by Bird and coworkers (15).

In the present investigation, it was found that chicks on a corn-soy bean diet appeared to require some other factor in addition to vitamin B_{12} for maximum growth.

EXPERIMENTAL

Chick Assay—New Hampshire pullets and cockerels were placed on Diet 1 at 6 months of age. The birds were kept in a laying house either on a raised wire floor or on a sugar-cane litter which was changed at frequent intervals. After a depletion period of 2 weeks, the eggs laid by these pullets were incubated to serve as a source of chicks. These were placed in electrically heated battery brooders with ¾ inch mesh wire floors

in an air-conditioned room at 28° and were fed the experimental diets immediately. Diet 2 (Table I) was used as the deficient basal diet, and ten to twelve chicks were customarily placed in each group. They were weighed at frequent intervals, and growth and survival were used as criteria for estimating the potency of supplements during an assay period

TABLE I

Composition of Basal Diets

Ingredient	Diet 1 (hens)	Diet 2 (chicks)
	gm.	*gm.*
Soy bean meal, solvent process	30	70
Yellow corn-meal	57.8	23.1
Alfalfa meal	5	
Bone meal	4.2	
Calcium carbonate	2.3	2.0
Salt mixture*		1.7
Sodium chloride	0.7	0.8
Vitamin mixture in glucose (see below)	1.0	1.0
Corn oil + vitamins A, D, and E†		1.0
Vitamin A and D feeding oil	0.2	
Methionine (DL-)		0.3
Choline chloride		0.2
Vitamin mixture	*mg.*	*mg.*
Thiamine HCl		1
Riboflavin	1	1
Niacinamide	5	5
Pyridoxine	1	1
Calcium pantothenate	5	5
Pteroylglutamic acid	1	0.2
1-Acetoxy-2-methyl-4-naphthyl sodium phosphate		0.5
Biotin		0.02
Glucose	To 1 gm.	To 1 gm.

* The salt mixture is composed of the following in gm.: bone ash 1000, K_2HPO_4 200, KH_2PO_4 225, $MgSO_4$ 125, $MnSO_4$ (anhydrous) 40, ferric citrate 25, $CuSO_4 \cdot 5H_2O$ 1.0, KI 0.3, Zn acetate 0.7, $Al_2(SO_4)_3 \cdot 18H_2O$ 0.8, cobalt acetate 0.2, Ni carbonate 0.1.

† See reference (16) but with corn oil to 1 gm. rather than to 3 gm.

of 25 days. Supplements in certain cases were administered at weekly intervals by intramuscular injection or were fed by pipette and in other cases were added to the basal diet.

Vitamin B_{12} Preparations—Concentrates were prepared from liver extract (15 units per ml.) by chromatography on silicic acid columns (17). A pink fraction was separated by this means which was purified by further chromatography and was found to give a characteristic absorption

spectrum with peaks at 273, 351, and 525 mμ. These peaks were thus at markedly different points from those reported for the crystalline anti-pernicious anemia factor (APA factor) at 278, 361, and 550 mμ respectively (18). Microbiological assay with *Lactobacillus leichmannii* 313 (19), with use of APA factor (17) or vitamin B_{12} (Cobione, Merck) as standards, was used to evaluate the potency of various preparations of this fraction. A solution, "Concentrate 1," of this fraction (vitamin B_{12b}) was selected for study with chicks. Its microbiological potency was found to correspond closely with the absorption value measured at 535 mμ, when a value of 54 is taken for the extinction coefficient ($E_{1\,cm.}^{1\%}$) of the crystalline factor (18). Concentrate 1 was adjusted to various appropriate dilutions so that an injection of 0.1 ml. supplied the requisite weekly dose. In some experiments, crystalline APA factor (Glaxo) or vitamin B_{12} (Cobione, Merck) were used as supplements.

Fermentation Materials—The organism *Streptomyces aureofaciens* (20) was grown in deep aerobic culture and the resulting material was studied for "animal protein factor" activity.[1] The entire fermented mash was fed in some experiments and in others a fraction (Fraction 1) was prepared by removing the soluble portion of the culture and drying the mycelium together with some filter aid. Fraction 1 was assayed microbiologically with *Lactobacillus leichmannii* 313, and various batches were found to have a potency corresponding to 0.4 to 1.0 γ of vitamin B_{12} per gm. The potency in the chick assay appeared to be appreciably higher, equivalent to about 4 γ per gm.

RESULTS AND DISCUSSION

On the basal diet, mortality was high and the surviving chicks grew slowly. Anemia was not found. Growth was rapid and most of the chicks survived when an adequate supplement was administered promptly after hatching either by injection or by addition to the diet. Many of the chicks died if the supplementation was withheld for a few days and for this reason a period of depletion was not used.

Liver Extract 1 was a bulk 15 unit preparation. It was assayed microbiologically many times and was found to have a potency corresponding to about 25 γ of vitamin B_{12} per ml. In the chick assay, groups which received 0.6 ml. of Liver Extract 1 usually reached an average weight of 200 to 220 gm. at 25 days. Higher levels of Liver Extract 1 produced only slightly heavier weights, while in experiments which are not summarized in this report, levels of 0.4 ml. or less resulted in lower weights.

With vitamin B_{12} or with Concentrate 1, the maximum average weight was in the neighborhood of 200 to 210 gm. at 25 days.

TABLE II

Response of Chicks on Diet 2 to Various Supplements

Twelve birds were used per group.

Supplement	Level in diet per kilo	Amount injected weekly	Weight and No of survivors (in parentheses) at 25 days		
			Experiment 1	Experiment 2	Experiment 3
			gm.	*gm.*	*gm.*
None.....			79 (2)	114 (3)	110 (3)
"			64 (1)	All dead	
Liver Extract 1		0.010 ml.	194 (12)		
" " 1		0.015 "	235 (12)		
" " 1	0.75 ml.			200 (10)	
" " 1	1.25 "			219 (10)	
" " 1	3.0 "			230 (11)	216 (12)
" " 1	3.0 "				
+ brewers' dried yeast...	100 gm.			193 (12)	
Liver Extract 1	3.0 ml.				
+ alfalfa meal	50 gm.			208 (12)	
Liver Extract 1	3.0 ml.				
+ dried distillers' solu- bles	50 gm.				230 (12)
Liver Extract 1.	3.0 ml.				
+ fish solubles	40 gm.				247 (11)
Crystalline APA factor*....		0.2 γ	177 (11)		
Concentrate 1, vitamin B_{12} equivalent to..		0.13 "		147 (9)	
" "		0.33 "		211 (10)	
" "		0.2 "			179 (12)
" "		0.4 "			208 (12)
" "		0.26 "†			158 (9)
" "		0.53 "†			203 (11)
Dried *S. aureofaciens* mash..	2 gm.				155 (7)
" " " " ..	6 "				222 (12)
" " " " ..	20 "				270 (10)
" " " " ..	60 "				277 (12)

* Glaxo Laboratories.

† Fed by pipette once weekly.

The results in Table II illustrate some of these points, and also show that the addition of yeast, alfalfa meal, distillers' solubles, or fish solubles produced little or no increase in growth beyond that obtained with liver extract. However, higher maximum weights were obtained with *Streptomyces aureofaciens* mash, 20 or 60 gm. per kilo of diet.

The results indicated that Concentrate 1 was utilized from 50 to 75 per cent as effectively by mouth as when injected into chicks. In patients with pernicious anemia it was found that, unless normal human gastric juice was also given, the oral administration of vitamin B_{12} was ineffective when 5 γ were given daily (21, 22), but a daily injection of 1 γ produced an excellent response in similar patients (23). The present results with chicks indicated that these animals were able to utilize vitamin B_{12} almost as effectively by mouth as when injected. This efficient utilization of orally administered vitamin B_{12} by presumably normal animals serves to emphasize the profound nutritional significance of the gastric lesion in pernicious anemia. This lesion may be presumed to be the

TABLE III

Experiment 4; Comparison of Effects of Supplements As Related to Composition of Diet (Ten Chicks per Group)

Supplement	Level in diet per kilo	Weight and No of survivors (in parentheses) at 25 days	
		Diet 2	Diet 3[*]
		gm.	*gm.*
None.....................................		103 (3)	162 (9)
S. aureofaciens, Fraction 1...............	2.5 gm.	140 (7)	239 (10)
" " " 1...............	5.0 "	210 (10)	240 (10)
" " " 1...............	10 "	230 (10)	261 (9)
" " " 1.....	30 "	271 (11)	266 (10)
Liver Extract 1.........................	0.3 ml.		202 (10)
" " 1.........................	0.6 "	209 (10)	
" " 1.........................	1.0 "	212 (9)	226 (10)

* Diet 3 differed from Diet 2 in that it contained 30 per cent soy bean meal and 63.1 per cent yellow corn-meal.

primary cause of the syndrome in human patients, inasmuch as the resultant absence of normal gastric juice appears to reduce greatly the uptake of vitamin B_{12} from the gut.

Assuming that 1.0 ml. of Liver Extract 1 per kilo of diet furnished sufficient vitamin B_{12}, the results in Table III constitute further evidence of the presence of an auxiliary growth factor in the *Streptomyces aureofaciens* fermentation material. Other results in Table III supply confirmation of the observation of Rubin and Bird (10) who noted that a deficiency of the "cow manure factor" in chicks was accentuated by increasing the level of soy bean meal in the diet.

Further results are summarized in Table IV, indicating that the vitamin B_{12} requirement of chicks on Diet 2 was satisfied by 0.3 γ of the crystalline material injected weekly or by 15 γ per kilo of diet. Again,

growth with vitamin B_{12} alone was slower than when the *Streptomyces aureofaciens* fermentation products were fed; evidently vitamin B_{12} did not remedy completely the deficiency of the basal diet. These results indicate that in terms of the experimental conditions encountered the "animal protein factor" consisted of vitamin B_{12} plus some factor as yet unidentified. In other experiments, a residue obtained by hot water extraction of beef and pork livers has been found to furnish the supplementary factor.

TABLE IV

Comparative Effects of Vitamin B_{12} and S. Aureofaciens Fractions

Supplement	Level in diet per kilo	Amount injected weekly	Weight and No of survivors (in parentheses) at	
			25 days	21 days
			Experiment 5	Experiment 6
		γ	*gm.*	*gm.*
None...........			129 (3)	136 (4)
Vitamin B_{12}*........		0.3	230 (11)	
" "		0.5	242 (11)	
" "	15 γ			163 (11)
" "	30 "			182 (12)
" " ....·..... ...	50 "			186 (11)
Dried *S. aureofaciens* mash.....	23 gm.			222 (12)
S. aureofaciens, Fraction 1.....	30 "		288 (10)	

* Merck and Company, Cobione.

The presence of vitamin B_{12} activity in culture broths of several species of *Streptomyces* was noted by Rickes and coworkers (24), who reported the isolation of crystalline vitamin B_{12} from the culture broth of a strain of *Streptomyces griseus*.

It was noted by Nichol *et al.* (25) that a vitamin B_{12} concentrate produced growth in chicks as rapid as that obtained by adding liver extract or fish solubles to the diet. However, the chicks on their basal diet reached the comparatively high weight of 237 gm. at 4 weeks, which would indicate that a more acute deficiency was encountered in the present investigation.

SUMMARY

1. A method for the biological assay of "animal protein factor" with chicks is described.

2. A level of 15 γ per kilo of diet or 0.3 γ injected intramuscularly each

week was found to supply the vitamin B_{12} requirement of the chicks, but maximum growth was not obtained until certain other supplements were added. Yeast, alfalfa meal, distillers' solubles, or fish solubles had little or no effect in this regard.

3. A fraction from *Streptomyces aureofaciens* cultures was found to have a higher potency for chicks than for *Lactobacillus leichmannii* as a source of vitamin B_{12}.

4. The oral potency of vitamin B_{12} was found to be 50 per cent that of the injected potency as estimated by the effects of a concentrate administered weekly. This efficient utilization of the orally administered vitamin in normal animals is contrasted with the reports of its inefficient use by patients with pernicious anemia.

BIBLIOGRAPHY

1. McFarlane, W. D., Fulmer, H. L., and Jukes, T. H., *Biochem. J.*, **24**, 1611 (1930).
2. van der Hoorn, R., Branion, H. D., and Graham, W. R., Jr., *Poultry Sc.*, **14**, 285 (1935).
3. Nestler, R. B., Byerly, T. C., Ellis, N. R., and Titus, H. W., *Poultry Sc.*, **15**, 67 (1936).
4. Christiansen, J. B., Deobald, H. J., Halpin, J. G., and Hart, E. B., *Poultry Sc.*, **18**, 481 (1939).
5. Hammond, J. C., and Titus, H. W., *Poultry Sc.*, **23**, 49 (1944).
6. Patton, A. R., Marvel, J. P., Petering, H. C., and Waddell, J., *J. Nutr.*, **31**, 485 (1946).
7. Scott, M. L., Norris, L. C., and Heuser, G. F., *J. Biol. Chem.*, **167**, 261 (1947).
8. Stokstad, E. L. R., Page, A. C., Pierce, J., Franklin, A. L., Jukes, T. H., Heinle, R. W., Epstein, M., and Welch, A. D., *J. Lab. and Clin. Med.*, **33**, 860 (1948).
9. Whitson, D., Hammond, J. C., Titus, H. W., and Bird, H. R., *Poultry Sc.*, **24**, 408 (1945).
10. Rubin, M., and Bird, H. R., *J. Nutr.*, **34**, 233 (1947).
11. Whitson, P., Titus, H. W., and Bird, H. R., *Poultry Sc.*, **25**, 52 (1946).
12. McGinnis, J., and Carver, J. S., *Poultry Sc.*, **26**, 457 (1947).
13. Ott, W. H., Rickes, E. L., and Wood, T. R., *J. Biol. Chem.*, **174**, 1047 (1948).
14. Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, **176**, 1477 (1948).
15. Bird, H. R., Rubin, M., and Groschke, A. C., *J. Biol. Chem.*, **174**, 611 (1948).
16. Jukes, T. H., Stokstad, E. L. R., and Gilbert, K. C., *Poultry Sc.*, **27**, 434 (1948).
17. Parker, C. F. J., and Smith, E. L., *Biochem. J.*, **33**, 41 (1948).
18. Ellis, B., Petrow, V., and Snook, G. F., *J. Pharm. and Pharmacol.*, **1**, 60 (1949).
19. Hoffmann, C. E., Stokstad, E. L. R., Franklin, A. L., and Jukes, T. H., *J. Biol. Chem.*, **176**, 1465 (1948).
20. Duggar, B. M., *Ann. New York Acad. Sc.*, **51**, 175 (1948).
21. Berk, L., Castle, W. B., Welch, A. D., Heinle, R. W., Anker, R., and Epstein, M., *New England J. Med.*, **239**, 911 (1948).

22. Hall, B. E., Morgan, E. H., and Campbell, D. C., *Proc. Staff Meetings Mayo Clin.*, **24**, 99 (1949).
23. Hall, B. E., and Campbell, D. C., *Proc. Staff Meetings Mayo Clin.*, **23**, 584 (1948).
24. Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, **108**, 634 (1948).
25. Nichol, C. A., Robblee, A. R., Cravens, W. W., and Elvehjem, C. A., *J. Biol. Chem.*, **177**, 631 (1949).

PREPARATION OF PURIFIED HYALURONIDASE FROM BOVINE TESTIS

By MONROE E. FREEMAN, PEARL ANDERSON, MARIAN OBERG, and ALBERT DORFMAN*

(From the Department of Chemistry and Physics, Army Medical Department Research and Graduate School, Washington)

Numerous procedures for the extraction and partial purification of hyaluronidase from bovine testis have been reported in the literature (1–11), but, in nearly all cases, the procedures have not been extended to the production of highly purified preparations. Hahn (11) has given detailed directions for the preparation of small quantities of the highly purified enzyme, but the method is long, tedious, and feasible only for the preparation of small quantities. The recent wide interest in hyaluronidase and its possible relation to several physiological and medical problems has prompted this laboratory to investigate simpler and easier techniques for preparing the enzyme in sufficient quantity to facilitate investigations into the nature of the purified enzyme and its physiological behavior. Since other laboratories are engaged in similar experimentation, a simplified procedure for preparing hyaluronidase of fairly high purity is presented here in detail.

Many of the steps for purification that have been previously reported were critically examined, as well as the varying conditions, with respect to temperature, pH, concentrations, ionic strength, etc. These steps have been accordingly rejected or incorporated into the following recommended procedure. The supporting data for these numerous experiments and selections are too voluminous to be presented here. Only the details of the selected steps will be given. In general, the following steps were found to be the most advantageous in regard to economy of time, effort, and yields of active product: (1) extraction of fresh testicular tissue with acetic acid; (2) preliminary precipitation of the active fraction with ammonium sulfate; (3) dialysis and removal of inactive, water-insoluble material; (4) fractional precipitation with ammonium sulfate; (5) fractional precipitation with ethanol; (6) refractionation with ethanol; and (7) refractionation of low activity fractions with ammonium sulfate or ethanol.

* Present address, Department of Pediatrics, University of Chicago, Chicago.

EXPERIMENTAL

Methods

Units of hyaluronidase are reported as arbitrary units (Dorfman and Ott (12)). This unit is nearly the same as the unit of other laboratories, with similar conditions and reagents for assay (13). It should be noted, however, that this "standard" unit was arbitrarily selected from an early enzyme preparation in this laboratory. A constant value of this standard unit has been maintained by frequent comparison of new preparations with the original standard.

Total nitrogen was determined by micro-Kjeldahl digestion and direct nesslerization, ammonia nitrogen by direct nesslerization. Approximate protein nitrogen was estimated by the difference of total and ammonia nitrogen. Exact protein determinations were carried out by micro-Kjeldahl digestion and nesslerization of dialyzed samples.

Preparation of Hyaluronidase

Extraction with Acetic Acid—Frozen bovine testes[1] were partly thawed, decapsulated, and minced by machine. The cold mince was rapidly stirred by motor for 1 hour with an equal weight of 0.1 N cold acetic acid and allowed to stand overnight at 5°. The material was centrifuged at 5000 R.P.M. for 15 minutes. The residue was extracted two times more with one-third the original weight of 0.1 N acetic acid. The additional extractions increased the yield of crude enzyme about 15 per cent. Extracts were combined and filtered with suction through a half inch pad of filter paper pulp. The filtrate, 2 liters per kilo of fresh tissue, contained about 800,000 units of hyaluronidase with a purity index of 250 units of enzyme per mg. of nitrogen.

Preliminary Precipitation with Ammonium Sulfate—212 gm. of solid ammonium sulfate were stirred rapidly into each liter of crude extract and allowed to stand a short time at 5°. The voluminous inactive precipitate was removed by gravity filtration and discarded. The clear filtrate was stirred with an additional 282 gm. of ammonium sulfate and allowed to stand overnight. The active precipitate was separated from the inactive supernatant by siphon and gravity filtration. The crude precipitate from each kilo of tissue was dissolved completely in 150 to 200 ml. of water. The solution contained at least 90 per cent of the enzyme extracted by the acetic acid, with a purity index of 800 to 1200 units per mg. of nitrogen.

Dialysis—After 12 to 24 hours dialysis against running tap water, cooled to 5°, a voluminous inactive fraction flocculated and was separated

---

[1] Frozen bovine testes were supplied through the courtesy of Armour and Company, Chicago, Illinois.

by suction filtration through paper pulp. Low temperature was essential during dialysis, since the enzyme at this stage was unstable in water suspension at room temperature. 20 to 40 per cent losses may be anticipated if the dialysis is conducted at room temperature.

Fractional Precipitation with Ammonium Sulfate—250 gm. of solid ammonium sulfate were rapidly stirred into each liter of dialyzed filtrate. Refrigeration was not essential when crude enzyme preparations were in contact with high concentrations of ammonium sulfate. The suspension was rapidly filtered through paper pulp to yield a water-clear filtrate. Occasionally, voluminous precipitates required one or more changes of filter pads. Cloudy filtrates were rapidly passed again through a small thick (1 inch) filter. Filter pads containing the precipitates were reserved for extraction. The clear filtrate was treated with additional 50 gm. of ammonium sulfate per liter, increasing the salt concentration to 26 per cent. The resulting precipitate was removed as described above. Additional precipitates were successively removed at 28.5, 31, and 33.3 per cent ammonium sulfate.

The filter pad and precipitate from each fraction were immediately mixed (Waring blendor) with 0.015 M phosphate-citrate buffer, pH 5.0, containing 0.9 per cent sodium chloride. The thick pulp suspension was poured into a Büchner funnel, drained, and well packed by suction. The cloudy filtrate was clarified by pouring again through the packed filter pad. The filter paper was extracted a second time with a minimum quantity of buffer. The extracts were combined and assayed for ammonia nitrogen, protein nitrogen, and hyaluronidase.

It has not been feasible to reproduce rigidly the exact conditions of each batch of tissue treated; consequently, the distribution of enzyme in the several ammonium sulfate fractions has varied with different runs. However, the general pattern was clear and is illustrated in Table I, which indicates the general distribution and yields that may be normally expected. The values (Table I) are averages of three typical batches, each totaling about 7,000,000 units of enzyme, each extracted from about 9 kilos of fresh tissue.

About 44 per cent of the enzyme in the acetic acid extracts was recovered with a purity index of 2000 units per mg. of protein nitrogen, a 2½-fold increase in the enzyme to nitrogen ratio, and was reserved for ethanol fractionation. Another 23 per cent with somewhat lower activity (1400 and 1070 units per mg. of N) was recovered in the 31 and 23 per cent ammonium sulfate fractions. These fractions of lower activity were combined and carried through the ethanol fractionation separately. These lower grade fractions usually required several additional fractionation steps and, therefore, were not pooled with the best fractions.

Ethanol Fractionation—It was found to be very important to maintain

temperatures below 0° whenever the enzyme was in contact with ethanol. To this end, the fractionation with ethanol was carried out in 8 liter bottles, Büchner funnels, and suction flasks immersed in a refrigerated bath at −5°. The suspensions and filtrates were stirred by motor and transferred by air pressure.

All available fractions from the ammonium sulfate treatment that had an activity near 2000 units per mg. of nitrogen were diluted to a concentration of approximately 0.16 mg. of N per ml. with 0.015 M phosphate-citrate buffer, pH 5.0, containing 0.9 per cent NaCl. The solution was cooled to −5°; and ethanol, previously cooled in an alcohol-CO_2 bath, was slowly added with rapid stirring (motor) to a concentration of 0.1 mole fraction. After standing for 1 hour, the slightly turbid suspension was pumped by air pressure onto a paper pulp filter and the suction flask immersed in a refrigerated bath. The water-clear filtrate was returned to

TABLE I

Ammonium Sulfate Fractionation of Crude Hyaluronidase Extracts

Concentration of $(NH_4)_2SO_4$	Total units of hyaluronidase recovered per kilo tissue	Hyaluronidase recovered from HAc extract	Purity index; units of enzyme per mg. N
per cent		*per cent*	
20	45,000	6	460
23	47,000	6	1400
26	126,000	17	2400
28.5	207,000	27	2020
31	130,000	17	1070
33.3	22,000	3	630

the mixing carboy and ethanol was again added to the concentration of 0.15 mole fraction. Additional precipitates were obtained in a similar manner at 0.20, 0.25, and 0.30 mole fractions of ethanol. After each filtration the paper pulp pads containing the precipitates were immediately immersed in 0.015 M phosphate-citrate buffer solution containing 0.2 per cent sodium chloride and extracted two times as described for the ammonium sulfate fractionation. A low temperature must be maintained until the active fractions have been dissolved in the phosphate-citrate buffer. The alcoholic suspensions at 0.1, 0.15, and 0.20 mole fractions were often gummy and rapidly clogged the filter pads, necessitating one or more fresh filter pads to maintain rapid filtration. With properly prepared filters of optimum thickness, 4 liters of the alcoholic suspension could be filtered in an hour or less.

The precipitates, dissolved in phosphate-citrate buffer, were analyzed for ammonia nitrogen, protein nitrogen, and hyaluronidase. As noted

above, all the detailed conditions were not rigorously standardized for successive batches; therefore, the distribution of yields and enzyme purity were not precisely reproduced in the several fractionations. However, they did show reasonable agreement and the averaged data from five different fractionations are shown in Table II. Each batch was about 4 liters of enzyme solution in 0.015 M phosphate-citrate buffer containing

TABLE II

*First Ethanol Fractionation of Hyaluronidase Solution**

Concentration of ethanol	Total enzyme recovered		Purity index; units of enzyme per mg. N
mole fraction	*units per l.*	*per cent*	
0.10	13,000	4	800
0.15	70,000	20	2000
0.20	133,000	38	5000
0.25	84,000	24	8200
0.30	8,000	2.5	1600

* Enzyme suspended in 0.015 M phosphate-citrate buffer at pH 5.0 containing 0.16 mg. of protein nitrogen per ml., 0.9 per cent NaCl, 350 units of enzyme per ml., and about 2000 units of hyaluronidase per mg. of nitrogen.

TABLE III

*Second Ethanol Fractionation of Hyaluronidase**

Concentration of ethanol	Total enzyme recovered		Purity index; units of enzyme per mg. N
mole fraction	*units per l.*	*per cent*	
0.10	71,000	7	6,600
0.15	580,000	58	16,000
0.20	190,000	19	8,000
0.25	120,000	12	5,200
0.30	9,000	1	1,200

* This enzyme was derived from the 0.25 mole fraction precipitate of the first ethanol treatment and was suspended in 0.015 M phosphate-citrate buffer at pH 5.0, containing 0.16 mg. of protein N per ml., 0.2 per cent NaCl, 990 units of enzyme per ml., and about 8200 units of enzyme per mg. of nitrogen.

0.9 per cent NaCl, at pH 5.0, and a protein nitrogen content of 0.16 mg. per ml. Each liter contained about 350,000 units of hyaluronidase, 2000 units of enzyme per mg. of nitrogen.

The precipitates from the first alcohol fractionation were pooled according to their purity index and refractionated with ethanol in the same way. The best fractions, 8200 units per mg. of nitrogen, were diluted to about 0.16 mg. of protein nitrogen per ml., 990 units of enzyme per ml., and 0.2 per cent NaCl. Typical data, Table III, show that 58 per cent

of the enzyme precipitated at a purity index of 16,000 units of enzyme per mg. of nitrogen. The lower grade fractions at 8000 and 5200 units per mg. of nitrogen were pooled with comparable fractions from other runs and reworked with ammonium sulfate and ethanol.

When the first ethanol precipitates at 6000 units per mg. of nitrogen were treated with ethanol a second time, the recoveries, Table IV, were not encouraging. This solution was diluted to 0.13 mg. of protein nitrogen per ml., 730 units of enzyme per ml., and 5600 units of enzyme per mg. of nitrogen, and fractionated with ethanol. Only 17 per cent of this sample showed an appreciable increase in purity, 5600 to 9100 units per mg. of N, while 64 per cent precipitated at 5000 units per mg. of N, the same ratio as the starting material. Similar results with other batches indicate that the lower grade fractions could not be significantly improved

TABLE IV

*Second Ethanol Fractionation of Low Activity Hyaluronidase**

Concentration of ethanol	Total enzyme recovered		Purity index; units of enzyme per mg N
mole fraction	*units per l.*	*per cent*	
0.10	34,000	45	2500
0.15	476,000	64.0	5000
0.20	122,000	16.5	9100
0.25	4,700	0.5	1000
0.30	1,100	0.1	500

* Lower grade fractions from the 0.2 mole fraction precipitate of the first ethanol treatment, suspended in 0.015 M phosphate-citrate buffer at pH 5.0 containing 0.13 mg. of protein nitrogen per ml., 0.2 per cent NaCl, 730 units of enzyme per ml., and about 5600 units per mg. of nitrogen.

by more than two successive ethanol treatments. Therefore, a second ammonium sulfate treatment was applied to the lower grade fractions after one or two ethanol treatments. The enzyme fractions that showed no improvement in the second ethanol treatment, Tables III and IV, were combined and suspended in the phosphate-citrate buffer and fractionated with ammonium sulfate. The results of this second salt fractionation following two ethanol treatments are shown in Table V. 20 per cent of the material was recovered at 18,000 units per mg. of nitrogen, a 3-fold increase in enzyme nitrogen ratio, and another 40 per cent increased only from 5600 to 5800 units per mg. of nitrogen.

By application of these techniques, 10 to 15 per cent of the enzyme originally extracted by dilute acetic acid was obtained that had an average purity index of 18,000 units per mg. of nitrogen. These fractions were dialyzed against ice water and lyophilized without significant loss

of activity. Small aliquots have been successfully purified to 65,000 units of hyaluronidase per mg. of nitrogen. However, these stages in purification were more conveniently carried out with refrigerated centrifugation and more careful manipulation of the ionic strength. Experiments with the latter technique are under investigation and will be described in another communication.

TABLE V

Second Ammonium Sulfate Fractionation of Hyaluronidase*

Concentration of $(NH_4)_2SO_4$	Total enzyme recovered	Total enzyme recovered	Purity index; units of enzyme per mg. N
per cent	*units per l.*	*per cent*	
16.6	25,000	2.0	5,100
19.0	40,000	3.5	6,300
26	144,000	12.5	3,100
28.5	448,200	39.0	5,800
31.0	234,500	20.0	18,000
33	61,700	5.0	7,800

* These samples included selected fractions, 5000 to 6000 units per mg. of nitrogen, that had been through one ammonium sulfate and two successive ethanol fractionations. They were resuspended in 0.015 M phosphate-citrate buffer at pH 5.0, containing 1100 units of enzyme per ml., 0.2 mg. of protein nitrogen per ml., and 5600 units of enzyme per mg. of nitrogen.

SUMMARY

Optimum conditions for the purification of hyaluronidase from bovine testis in a procedure of alternate ammonium sulfate and ethanol fractional precipitations have been ascertained. Low temperatures, low protein concentration, and low ionic strength in the ethanol treatments were found to be most advantageous in handling quantities of this material.

The first extract, 0.1 N HAc, of fresh tissue gave 750,000 to 1,000,000 units of enzyme, with an activity of 250 units per mg. of nitrogen. Four successive fractionations yielded about 10 per cent of the enzyme with an activity of almost 20,000 units per mg. of nitrogen, an 80-fold increase in purity. Increased yields were obtained by additional treatment of the lower grade fractions.

BIBLIOGRAPHY

1. Aylward, F. X., *Proc. Soc. Exp. Biol. and Med.*, **36,** 477 (1937).
2. Chain, E., and Duthie, E. S., *Brit. J. Exp. Path.*, **1,** 324 (1940).
3. Claude, A., and Duran-Reynals, F., *J. Exp. Med.*, **65,** 661 (1937).
4. Duran-Reynals, F., *J. Exp. Med.*, **50,** 327 (1929).
5. Morgan, W. T. J., and McClean, D., *J. Soc. Chem. Ind.*, **51,** 912 (1932).

6. Madinaveitia, J., *Biochem. J.*, **32,** 1806 (1938).
7. Madinaveitia, J., *Biochem. J.*, **33,** 347 (1939).
8. Madinaveitia, J., *Biochem. J.*, **33,** 1470 (1939).
9. Madinaveitia, J., *Biochem. J.*, **35,** 447 (1941).
10. Perlman, P. L., Leonard, S. L., and Kurzrok, R. S., *Endocrinology*, **42,** 26 (1948).
11. Hahn, L., *Biochem. Z.*, **315,** 83 (1943).
12. Dorfman, A., and Ott, M. L., *J. Biol. Chem.*, **172,** 367 (1948).
13. Warren, G. H., Durso, J. G., and Levin, N. R., *Endocrinology*, **43,** 48 (1948).

THE INTERMEDIARY METABOLISM OF PHENYLALANINE LABELED WITH RADIOACTIVE CARBON*

By BERNARD SCHEPARTZ† AND SAMUEL GURIN

(*From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia*)

The metabolic formation of ketone bodies from phenylalanine and tyrosine has been a recognized phenomenon for many years (1). Since homogentisic acid is formed from tyrosine (2) and phenylalanine (3), and since it in turn forms ketone bodies (1), it has been considered to be a probable intermediary metabolite in the production of ketone bodies from phenylalanine and tyrosine. However, the pathway by which tyrosine is converted to homogentisic acid and the mechanisms whereby the aromatic ring is finally cleaved to form acetoacetic acid have remained obscure.

With the advent of tracer techniques it has become possible to study these metabolic pathways more directly and to obtain more information about the intermediate steps involved. For this purpose DL-phenylalanine containing C^{14} in definite positions in the benzene ring has been synthesized and investigated in biological systems. Data are also presented on the metabolism of DL-phenylalanine labeled with C^{14} in the side chain.

Experimental Procedures

Synthesis of DL-Phenylalanine Labeled with C^{14} in Carboxyl and α Positions—This preparation was described briefly in a previous communication from this Department (4). Radioactive barium carbonate (50 mM) was converted to acetylene and acetaldehyde, in general according to the procedure of Cramer and Kistiakowsky (5). The doubly labeled acetaldehyde was oxidized to acetic acid with an excess of silver oxide and isolated as the sodium salt in an over-all yield of 40 per cent from the barium carbonate. The salt was converted to ethyl bromoacetate (86 per cent yield) by the method of Auwers and Bernhardi (6). Glycine was then prepared (7) and benzoylated without isolation to give a 56 per cent yield of hippuric acid

from the bromo ester. The standard azlactone procedure (8, 9) afforded a 50 per cent yield of phenylalanine from the hippuric acid.

Synthesis of DL-*Phenylalanine Labeled with* C^{14} *in Positions 1, 3, and 5 of Benzene Ring*—The course taken by the isotopic carbon atoms in this synthesis is indicated by asterisks in Scheme 1. Carboxyl-labeled sodium acetate was prepared in 73 per cent yield from 50 mM of isotopic barium carbonate, as described by Sakami, Evans, and Gurin (10). Distillation of a mixture of the sodium acetate with excess phosphorus tribromide produced acetyl bromide in 72 per cent yield. Pyruvonitrile was then prepared in 60 per cent yield by the procedure of Tschelinzeff and Schmidt (11). According to the directions of Anker (12), the nitrile was hydrolyzed to the amide and then to pyruvic acid, in yields varying from 40 to 70

$$
\overset{*}{Ba}CO_3 \quad CH_3\overset{*}{C}OONa \rightarrow CH_3\overset{*}{C}OBr \rightarrow CH_3\overset{*}{C}OCN \rightarrow CH_3\overset{*}{C}OCONH_2
$$

CH₃   COOH

CH₃

CH₂COCOOH

HOOC *COOH HOOC* *COOH

COOC₂H₅

CH   CH₂Br   CH₂CNHCOCH₃   CH₂CHNH₂COOH

|
COOC₂H₅
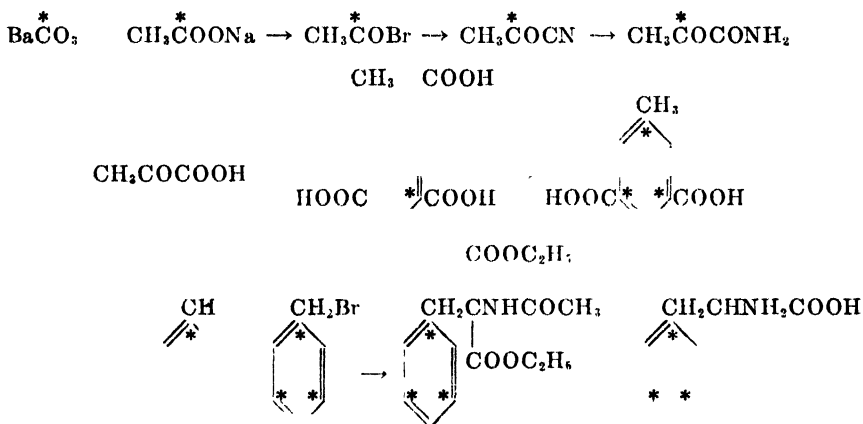
SCHEME 1. Synthesis of phenylalanine labeled in the ring

per cent. The pyruvic acid underwent cyclization (13) in 50 per cent sodium hydroxide solution to methyldihydrotrimesic acid (55 per cent yield) which was converted to uvitic acid by heating with concentrated sulfuric acid (yield 91 per cent). After dilution of the product with non-isotopic uvitic acid, toluene[1] was obtained in yields of 70 to 100 per cent by fusion with soda lime (14). The toluene was treated with bromine in carbon tetrachloride to give benzyl bromide in a 71 per cent yield (15). Phenylalanine was then prepared by means of acetamino-malonic ester by procedures recently described (16, 17). The yield of ring-labeled phenylalanine from benzyl bromide was 40 per cent.

---

[1] While this paper was in preparation, a description of a similar procedure for converting pyruvic acid to toluene was published (Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. F., Isotopic carbon, New York, 228 (1949)).

Procedures with Animals—In the two experiments reported on intact rats, glucosuria was produced by the daily, subcutaneous injection of phlorhizin in oil at a dosage of 120 to 200 mg. per kilo. The rats were fasted for 24 hours before the series of injections and throughout the rest of the experiment. When considerable glucose and ketone bodies were being excreted, the phenylalanine to be studied was injected intraperitoneally in divided doses (total 100 mg.).

For the collection of respiratory carbon dioxide, the rats were placed in a vessel which was aerated with a stream of carbon dioxide-free air, the effluent gases passed through a solution of sodium hydroxide, and barium carbonate formed at the end of the collection period by addition of barium chloride. Urine samples were preserved with toluene.

CH₃COCH₂COOH
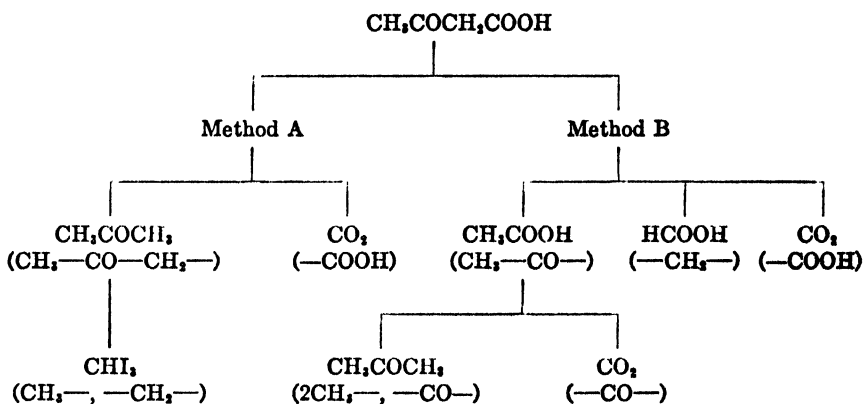
SCHEME 2. Degradation of acetoacetate

Procedures with Liver Slices—Slices 0.5 mm. thick were cut with the Stadie slicer (18) from the livers of rats killed by stunning and decapitation. The suspension medium used was that described by Krebs (19), except that magnesium sulfate was replaced by magnesium chloride. In general, 20 ml. of medium were used for each 2.5 gm. (wet weight) of liver slices. The gas phase was oxygen, and the bath temperature 38°.

Degradation of Acetoacetic Acid; Method A—Acetoacetic acid was decomposed into a carboxyl fraction and an acetone fraction (Scheme 2) by the method of Van Slyke (20) with some modifications. Rat urine or the supernatant medium decanted from the liver slices was clarified by treatment with a solution of copper sulfate and a suspension of calcium hydroxide. Following centrifugation, the supernatant solutions were acidified to pH 3 and aerated to remove dissolved carbon dioxide. (All aerations mentioned in this paper were performed with a stream of air freed of carbon dioxide by passage through a tower of soda lime.) After

addition of mercuric sulfate and sulfuric acid, the solutions were refluxed while being continuously aerated. The acetone fraction representing the last 3 carbon atoms of the acetoacetate was precipitated as the mercury complex, while the carboxyl fraction, liberated as carbon dioxide, was trapped in barium hydroxide. All acetone samples were purified by solution of the mercury complex in dilute hydrochloric acid, steam distillation of the acetone, and reprecipitation with mercuric sulfate.

In some cases sufficient acetone was available for further degradation by distillation into alkaline hypoiodite, resulting in iodoform derived from the two methyl groups (the original methyl and methylene groups of acetoacetate).

No data are reported for β-hydroxybutyric acid, because the dichromate oxidation used in degrading this substance by the Van Slyke method was found to oxidize radioactive impurities.

Method B—For this degradation the procedure of Weinhouse and Millington (21) was extended in order to obtain additional data from the acetate fraction (Scheme 2). The supernatant medium from experiments with liver slices was treated with copper sulfate and calcium hydroxide and freed of dissolved carbon dioxide as in Method A. The residual solution was then cooled in an ice bath, acidified with sulfuric acid, and treated with a slight excess of potassium permanganate (calculated from the amount of acetoacetate present). Under these conditions the acetoacetic acid was decomposed into carbon dioxide (from the carboxyl group), formic acid (from the methylene carbon atom), and acetic acid (from the carbonyl and terminal methyl carbon atoms). In the experiments reported in this paper, substances were present which gave rise to false radioactivity in the first two fractions (carboxyl and formate), so that only the acetate fraction was utilized.

After aeration to remove the carbon dioxide arising from the carboxyl group, the excess permanganate was destroyed with hydrogen peroxide and the volatile acids distilled from the mixture. The formic acid in the distillate was then oxidized to carbon dioxide by refluxing with a solution of mercuric sulfate, the carbon dioxide being removed by aeration as before. The remaining acetic acid was distilled, characterized by its Duclaux constants, and isolated as the barium salt. Usually the yield of acetic acid was quantitative.

By pyrolysis in a sealed, evacuated tube, the barium acetate was converted into barium carbonate (representing the original carbonyl group of the acetoacetate) and acetone (differing from the acetone of Method A in that both methyls are derived from the terminal methyl group of the acetoacetate). Both fractions from the pyrolysis were purified before being assayed for radioactivity.

Measurement of Radioactivity—All organic compounds were oxidised with the wet combustion mixture of Van Slyke and Folch (22), modified by the inclusion of a small amount of selenium. Samples were assayed for radioactivity in the form of barium carbonate, spread in a uniform layer on a met l planchet. A thin mica window Geiger-Müller counter (2.5 mg. per sq. cm.) was used. After subtracting background counts, the activities of the samples were corrected to the values at infinite thickness by means of a correction curve similar to the one described by Reid (23). The final values were expressed as counts per minute per mg. of carbon. Suffi ient counts were taken on most samples to reduce the standard deviation to 5 per cent or less.

<div align="center">TABLE I</div>

Experiment 2. Incubation of Liver Slices with Phenylalanine Labeled in Side Chain

Substance	Carbon atoms of acetoacetate represented	Counts per min. per mg C
Phenylalanine used.......		24.3
Respiratory CO_2		6.3
Degradation of acetoacetate, Method A		
CO_2..	—COOH	10.6
Acetone......	—CH_2—, —CO—, CH_3—	0.0

<div align="center">*Results*</div>

Experiments with Phenylalanine Labeled in Side Chain—In a preliminary experiment (Experiment 1) with DL-phenylalanine containing C^{14} in the carboxyl and α positions, 100 mg. of the amino acid were administered to a phlorhizinized rat, weighing 199 gm., over a period of several hours. Low radioactivity was found in the respiratory carbon dioxide of the first 6 hours and in some of the fractions of the urinary ketone bodies. The data suggested that the bulk of the C^{14} was located in the carboxyl carbon of the acetoacetic acid, but the low concentration of isotope prevented any definite conclusions.

Table I summarizes the data of Experiment 2, in which 5 mg. of the phenylalanine were incubated with 2.5 gm. (wet weight) of liver slices for 4 hours. At the end of the incubation the acetoacetate was degraded by Method A, after addition of non-isotopic sodium acetoacetate as a carrier. In comparing the radioactivity of the respiratory carbon dioxide with that of the acetoacetate, it must be borne in mind that the former did not suffer any dilution with added carrier (estimated to be a 5-fold dilution of the acetoacetate in this case). It is clear from the data that all of the radioactivity in the acetoacetate was localized in the carboxyl group.

Experiments with Phenylalanine Labeled in Ring—In Experiment 3, 100 mg. of DL-phenylalanine labeled with C^{14} in the 1, 3, and 5 positions of the benzene ring were administered to a phlorhizinized rat, weighing 260 gm., over a period of 2 hours. Respiratory carbon dioxide was collected during the 6th hour. The acetoacetic acid in the urine collected for 24 hours was degraded by Method A. Table II summarizes the re-

TABLE II

Experiment 3. Administration to Phlorhizinized Rat of Phenylalanine Labeled in Ring

Substance	Carbon atoms of acetoacetate represented	Counts per min. per mg C
Phenylalanine used		255
Respiratory CO_2, 6th hr.........		2.7
Degradation of acetoacetate, Method A		
CO_2	—COOH	0.76
Acetone....................	—CH_2—, —CO—, CH_3—	3.5

TABLE III

Incubation of Liver Slices with Phenylalanine Labeled in Ring

Substance	Carbon atoms of acetoacetate represented	Counts per min. per mg C	
		Experiment 4	Experiment 5
Respiratory CO_2................			7.1
Degradation of acetoacetate, Method A			
CO_2..........................	—COOH	0.0	1.0
Acetone	—CH_2—, —CO—, CH_3—	10.1	7.4
Iodoform.....	—CH_2—, CH_3—		7.3*

* Very little material was available for this sample, and no quantitative significance can be attached to the result.

sults. The bulk of the isotope was located in the last 3 carbon atoms of the acetoacetate.

These findings were confirmed by experiments performed *in vitro* (Table III). In Experiment 4, 2.6 gm. (wet weight) of liver slices were incubated for 3 hours with 10 mg. of phenylalanine, while in Experiment 5 the same amount of phenylalanine was used with 5 gm. of liver for 4 hours. Sodium butyrate (0.02 M) was present in the medium in both cases in order to provide sufficient acetoacetate for the degradation; so that the extent of dilution of the radioactive samples was not known. (These experiments

were performed before the addition of acetoacetate carrier was decided upon as a routine procedure.) Unfortunately, the value obtained for the iodoform was of qualitative significance only. However, the results provided further proof that the major site of the radioactivity was in the acetone fraction, while the carboxyl group contained little or no isotope.

For the purpose of establishing the location of the isotope more precisely, Experiments 6 and 7 were carried out (Table IV). In both cases 10 gm. of liver slices were incubated for 4 hours with 20 mg. of ring-labeled phenylalanine. Non-isotopic acetoacetate was added as carrier before the degradations, resulting in 10- and 9-fold dilutions, respectively. Degra-

TABLE IV

Degradation of Acetoacetate Derived from Ring-Labeled Phenylalanine

Substance analyzed	Carbon atoms of acetoacetate represented	Counts per min. per mg C	
		Experiment 6	Experiment 7
Respiratory CO_2		17.3	24.3
Degradation of acetoacetate, Method A			
CO_2....	—COOH	0.7	0.7
Acetone	CH_3—, —CO—, —CH_3—	7.4	10.0
Iodoform.........................	CH_3—, —CH_3—	10.6	9.4*
Degradation of acetoacetate by Method B			
Acetate.........................	CH_3—, —CO—	10.3	13.6
CO_2 from pyrolysis................	—CO—	0.3	0.5
Acetone from pyrolysis	CH_3—, CH_3—, —CO—	12.7	21.2

* See foot-note to Table III.

dations were carried out by Methods A and B in parallel. The activity of the iodoform fraction of Method A in Experiment 6 accounted for 95 per cent of the isotope in the acetone fraction, indicating that the bulk of the C^{14} was in the methylene or methyl carbon atom (or both) of the acetoacetate.[2] This result was confirmed by the low activity found in the carbon dioxide from the pyrolysis step of Method B in both experiments, representing the original carbonyl carbon atom of the acetoacetate. The acetate fractions of Method B in both cases accounted for almost all of the radioactivity observed in the acetone fractions of Method A (93 and 90

[2] If the iodoform had contained all of the isotope of the acetone, the specific activity per mg. of C of the former would have been 3/2 that of the latter.

$$\frac{-CH_3- + CH_3-}{2} \div \frac{CH_3- + -CO- + -CH_3-}{3} = 3/2 \text{ if } -CO- = 0$$

per cent, respectively[3]). Therefore the methylene carbon atom of aceto-acetate contained little or no activity. Since the carbonyl carbon atom of the acetoacetate had already been eliminated as a possibility, the bulk of the isotope could be assigned to the methyl carbon atom. Confirmation of this conclusion was afforded by a comparison of the radioactivities of the acetone fractions produced in Methods A and B in both experiments. The acetone of Method B differed from that of Method A in containing 2 of the original methyl carbon atoms instead of 1; hence the former acetone (Method B) would be expected to have twice the activity of the latter (Method A) if the chief site of the isotope were the methyl carbon atom.[4] This was indeed the case; therefore, the location of the heaviest C^{14} concentration was established as the methyl carbon atom of acetoacetate.

It may be of interest to record at this point that, despite the satisfactory incorporation of isotope from phenylalanine into acetoacetate in liver slices, no appreciable quantities of "extra" ketone bodies were formed under the conditions of these experiments in flasks containing phenylalanine compared with control flasks containing no substrate. The advantages of the isotopic tracer technique are thus manifest.

<div align="center">DISCUSSION</div>

In Scheme 3 there is presented a metabolic scheme for the incorporation into acetoacetic acid of the isotopic carbon atoms from the two types of labeled phenylalanine. The only pathway indicated for the conversion of phenylalanine to acetoacetic acid involves the preliminary formation of tyrosine, since other metabolites of phenylalanine, such as phenyl-pyruvic and phenyllactic acids, form no ketone bodies in the liver (24, 25) under conditions in which both phenylalanine and tyrosine are keto-genic (1, 25, 26).

From the results of the experiments with phenylalanine labeled in the side chain, it appears that the α-carbon atom of phenylalanine forms the carboxyl group of acetoacetate. This is in complete accord with the report

[3] If the acetate had contained all of the isotope of the acetone, the specific activity per mg. of C of the former would have been 3/2 that of the latter.

$$\frac{CH_3- + -CO-}{2} \div \frac{CH_3- + -CO- + -CH_2-}{3} = 3/2 \text{ if } -CH_2- = 0$$

[4] This may be shown as follows:

$$\frac{2CH_3- + -CO-}{3} \div \frac{CH_3- + -CO- + -CH}{3} = 2$$

approximately, if $-CH_2-$ and $-CO-$ are relatively small.

of Weinhouse and Millington (21) that the β-carbon atom of tyrosine is the precursor of the methylene carbon atom of acetoacetate. It is noteworthy that the isotope in our experiments is not distributed between the carbonyl and carboxyl groups of acetoacetate and that Weinhouse and Millington observed no randomization between the methyl and methylene groups. Since carboxyl-labeled octanoic (27, 28) and butyric acids (29) in liver slices form acetoacetate in which the isotope is considerably randomized, and since acetoacetic acid, once formed, does not cleave and randomly recondense in liver slices (28), it is apparent that the acetoacetate formed from the phenylalanine and tyrosine arises from a 4-carbon precursor, and not from the random condensation of 2-carbon fragments. This precursor is derived from 2 carbon atoms of the side chain and 2 of the adjacent carbon atoms of the ring and is transformed to acetoacetic acid without further scission of carbon-carbon bonds.
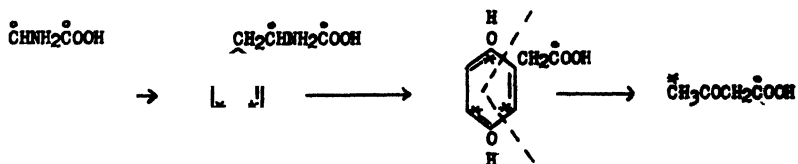
SCHEME 3. Origin of carbon atoms of acetoacetate. The asterisks and small circles, respectively, indicate the labeling of phenylalanine with C^{14} in separate experiments.

In the experiments with ring-labeled phenylalanine the presence of appreciable isotope in the respiratory carbon dioxide of both the intact rat and the liver slices is obvious proof of the cleavage of the aromatic ring, a point also confirmed by the radioactivity of acetoacetic acid, an acyclic metabolic product. Furthermore, there was again no redistribution of isotope between the methyl and methylene carbon atoms of the acetoacetate obtained after incubation with liver slices.

Since the C^{14} in this type of phenylalanine is located in positions 1, 3, and 5 of the ring, the formation of acetoacetate labeled in the terminal methyl group is evidence for a migration of the side chain from its original position on the ring (Scheme 3). The formation of ketone bodies from the ring-labeled phenylalanine without rearrangement of the side chain on the ring would result in carbonyl-labeled acetoacetate. Such a migration was postulated by Meyer (30) and Friedmann (31) to explain the positions of the hydroxyl groups of homogentisic acid as contrasted with the p-hydroxyl group of tyrosine. In our experiments the terminal (γ) carbon atom of the acetoacetate originates in carbon atom 1 or (more probably) in carbon atom 3 of the phenylalanine ring, while carbon atom 2 of the ring, "labeled" with non-radioactive carbon, is the precursor of the

carbonyl carbon atom of the acetoacetate. This evidence provides strong support for the belief that homogentisic acid is derived from tyrosine or p-hydroxyphenyl pyruvate by means of a migration of the side chain. The resulting acetic acid side chain of homogentisic acid is seen (Scheme 3) to be attached to a non-radioactive carbon atom. Cleavage of the resulting homogentisic acid (or quinone acetic?) would be expected to yield acetoacetate containing isotope in the terminal methyl position. All of our data are in complete accord with this mechanism.

It can be calculated from those experiments in which known amounts of carrier were used that the single, radioactive carbon atom of the phenylalanine (originating in the ring or side chain, respectively) responsible for the bulk of the radioactivity in the acetoacetate underwent an approximately 3-fold dilution in the course of metabolism. Conversely, approximately one-fourth of the acetoacetate produced by the slices must have come from the phenylalanine. Only a few per cent of the phenylalanine introduced into each flask, however, can be accounted for in the acetoacetate.

The fate of the remaining carbon atoms in the aromatic ring still remains to be clarified. Efforts in this direction are under way at the present time.

SUMMARY

Two types of radioactive DL-phenylalanine were synthesized. One was labeled with C^{14} in the carboxyl and α positions of the side chain, while the other preparation contained C^{14} in positions 1, 3, and 5 of the aromatic ring.

The conversion of both types of phenylalanine to acetoacetate in phlorhizinized animals as well as in liver slices was investigated. The α-carbon atom of phenylalanine was shown to be the precursor of the carboxyl carbon atom of acetoacetate.

Direct evidence was obtained for the shift of the side chain of phenylalanine prior to the formation of acetoacetate. The terminal carbon atom of acetoacetate was shown to come from carbon atom 1 or 3 of the ring of phenylalanine. The carbonyl carbon atom of acetoacetate is derived from carbon atom 2 of the ring.

It was demonstrated in several ways that the aromatic ring of phenyl-

alanine undergoes metabolic cleavage and can be oxidized to carbon dioxide.

The absence of significant randomization of isotope in the acetoacetate produced from phenylalanine indicates that a 4-carbon precursor of acetoacetate is involved. This precursor derives half of its carbon atoms from the side chain and the other half from the aromatic ring.

BIBLIOGRAPHY

1. Embden, G., Salomon, H., and Schmidt, P., *Beitr. chem. Physiol. u. Path.*, **8**, 129 (1906).
2. Wolkow, M., and Baumann, E , *Z. physiol. Chem.*, **15**, 228 (1891).
3. Falta, W., and Langstein, L., *Z. physiol. Chem.*, **37**, 513 (1903).
4. Gurin, S., and Delluva, A. M., *J. Biol. Chem.*, **170**, 545 (1947).
5. Cramer, R. D., and Kistiakowsky, G. B., *J. Biol. Chem.*, **137**, 549 (1941).
6. Auwers, K., and Bernhardi, R., *Ber. chem. Ges.*, **24**, 2209 (1891).
7. Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127**, 301 (1939).
8. *Org. Syntheses*, coll. **2**, 55 (1943).
9. *Org. Syntheses*, coll. **2**, 489 (1943).
10. Sakami, W., Evans, E. W., and Gurin, S., *J. Am. Chem. Soc.*, **69**, 1110 (1947).
11. Tschelinzeff, W., and Schmidt, W., *Ber. chem. Ges.*, **62**, 2210 (1929).
12. Anker, H. S., *J. Biol. Chem.*, **176**, 1333 (1948).
13. Wolff, L., *Ann. Chem.*, **305**, 125 (1899).
14. von Baeyer, A., *Z. Chem.*, **4**, 119 (1868).
15. Sampey, J. R., Fawcett, F. S., and Morehead, B. A., *J. Am. Chem. Soc.*, **62**, 1839 (1940).
16. Snyder, H. R., Shekleton, J. F., and Lewis, C. D., *J. Am. Chem. Soc.*, **67**, 310 (1945).
17. Albertson, N. F., and Archer, S., *J. Am. Chem. Soc.*, **67**, 308 (1945).
18. Stadie, W. C., and Riggs, B. C., *J. Biol. Chem.*, **154**, 687 (1944).
19. Krebs, H. A., *Z. physiol. Chem.*, **217**, 191 (1933).
20. Van Slyke, D. D., *J. Biol. Chem.*, **32**, 455 (1917).
21. Weinhouse, S., and Millington, R. H., *J. Biol. Chem.*, **175**, 995 (1948).
22. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136**, 509 (1940).
23. Reid, A. F., Preparation and measurement of isotopic tracers, Ann Arbor, **83** (1946).
24. Embden, G., and Baldes, K., *Biochem. Z.*, **55**, 301 (1913).
25. Edson, N. L., *Biochem. J.*, **29**, 2498 (1935).
26. Wakeman, A. J., and Dakin, H. D., *J. Biol. Chem.*, **9**, 139 (1911).
27. Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **155**, 143 (1944).
28. Buchanan, J. M., Sakami, W., and Gurin, S., *J. Biol. Chem.*, **169**, 411 (1947).
29. Medes, G., Weinhouse, S., and Floyd, N. F., *J. Biol. Chem.*, **157**, 35 (1945).
30. Meyer, F., *Deut. Arch. klin. Med.*, **70**, 443 (1901).
31. Friedmann, E., *Beitr. chem. Physiol. u. Path.*, **11**, 304 (1908).

CARBAMATES IN THE CHEMOTHERAPY OF LEUCEMIA

VII. THE RATE OF CATABOLISM OF URETHANE IN NORMAL AND NEOPLASTIC MICE*

By JACK H. MITCHELL, Jr., OLIVIA S. HUTCHISON, HOWARD E. SKIPPER, AND CARL E. BRYAN

(From the Southern Research Institute, Birmingham, Alabama)

In a recent paper (1) concerned with the retention of the carbonyl carbon from urethane by neoplastic animals it was observed that two mice with advanced spontaneous lymphoid leucemia, one mouse with advanced spontaneous mammary carcinoma, and one with advanced methylcholanthrene-induced mammary carcinoma expired considerably less of the labeled carbon than did normal mice over a 24 hour period. These neoplastic mice were all found to have a correspondingly higher tissue retention of the carbon atom in question. We have undertaken in this present paper to determine whether this higher retention is in the form of urethane or of a metabolite of this compound.

EXPERIMENTAL

Chemical Determination of Urethane—Urethane was determined by a procedure suggested by Boyland[1] which entailed hydrolysis with sodium hydroxide and subsequent estimation of the resulting alcohol by the method of Winnick (2). 1 ml. of 0.04 N potassium dichromate in 10 N sulfuric acid was pipetted into the inner ring of a Conway unit, and the solution to be assayed, usually 0.5 to 1.0 ml., along with 1.0 ml. of 40 per cent sodium hydroxide, was placed in the outer compartment. The unit was sealed immediately with the greased cover glass and rotated to mix the solutions in the outer ring. It was then placed in an oven maintained at 40° for 4 hours. This incubation period was adequate for hydrolysis and subsequent diffusion of the resulting alcohol. An excess of potassium iodide, 0.5 ml. of 1 M solution, was added to the inner ring of the diffusion unit and the liberated iodine was titrated with 0.02 N sodium thiosulfate solution. Blanks were run with each set of experiments and all analyses were made in duplicate or triplicate.

In order to test the accuracy of the method, a number of determinations were made with standard urethane solutions alone and in combination with blood.

Injection Procedure—The animals for radioactivity experiments were injected with 1400 mg. per kilo of carbonyl-labeled urethane containing an activity of 2 μc. Animals which were to be used in chemical studies were injected with 1400 mg. per kilo of non-radioactive urethane per animal. In these latter experiments blood samples from five to eight animals were collected at intervals over a 24 hour period for determination of the rate of disappearance of free urethane from the pooled blood.

Animals Used—Normal CFW strain white mice have been used as controls for the Akm strain mice with spontaneous lymphoid leucemia and

TABLE I

Results of Assays on Standard Urethane Solutions

Urethane present	Urethane found	Error
mg.	*mg.*	*per cent*
0.05	0.095	+90
0.05	0.074	+49
0.10	0.09	−10
0.10	0.12	+20
0.30	0.19	−36
0.30	0.21	−30
0.40	0.31	−22
0.40	0.34	−15
0.50	0.43	−14
0.50	0.47	−6
0.50	0.46	−8
1.00	0.96	−2
1.00	0.96	−4

CFW strain mice with spontaneous mammary carcinoma and methylcholanthrene-induced mammary carcinoma.

Radioactivity Assays—Assays for radioactivity were conducted as previously described (3).

Results

Chemical Determinations—Tables I and II show the results which were obtained when determinations were made with standard urethane solutions alone and in combination with blood. The accuracy of the method increases with increasing amounts of urethane up to a concentration of about 0.5 mg. The reproducibility of the results when 0.50 mg. of urethane is present is shown in Table III.

TABLE II

Results of Assays on Standard Urethane Solutions Plus 0.5 Ml. of Mouse Blood

Urethane present	Urethane found	Error
mg.	*mg.*	*per cent*
0.10	0.165	+64
0.10	0.152	+51
0.50	0.44	−12
0.50	0.49	−2
0.50	0.49	−2

TABLE III

Reproducibility of Urethane Determination

0.50 mg. of urethane present.

Urethane found	Error
mg.	*mg.*
0.62	+0.12
0.44	−0.06
0.46	−0.04
0.40	−0.10
0.53	+0.03
0.57	+0.07

$$\text{Average } 0.50 \pm \sqrt{\frac{0.0354}{6}} = \pm 0.08 \text{ mg.}$$

TABLE IV

Urethane in Blood and Tissues of Mice at Various Intervals after Injection with 1400 Mg. of Urethane per Kilo

Time after injection	Mg. urethane per 0.5 ml. blood			Mg. urethane per 0.5 gm. tissue			
	Leucemic	Normal	Mammary carcinoma	Leucemic liver	Normal liver	Mammary carcinoma	
						Tumor	Fetus
hrs.							
2½		0.78*			0.64*		
6	0.70*	0.66*		0.57*	0.53*		
8	0.44*	0.52*	0.45†			0.35†	0.42‡
24	0.05*	0.05*	0.18†	0.03*	0.02*		
24		0.08†	0.21†				
24		0.02‡	0.37†				
24		0.07†	0.17†				

* Five to eight mice were used; average value.
† Two mice were used; average value.
‡ One mouse was used.

Disappearance of Urethane from Blood Stream Following Injection—
Following the injection of 1400 mg. per kilo of urethane per animal into
groups of five to eight normal mice, the blood of each group was pooled at
intervals over 24 hour periods. In Table IV are given the results of ure-
thane determinations in 0.5 ml. of blood by the microdiffusion method.

Table IV also compares the urethane values found at several time in-
tervals in blood and several tissues of normal mice with that found in
mice with advanced spontaneous lymphoid leucemia and with advanced
spontaneous mammary carcinoma. In the latter, four separate trials
for the 24 hour period were made with groups of two animals each, the
blood from the two being pooled in each instance for the analysis.

Radioactive Determinations— Table V gives the data which offer a com-
parison between the retention of radioactive carbon from carbonyl-labeled

TABLE V

*Retention of Radioactivity from Carbonyl-Labeled Urethane by Mouse with Methyl-
cholanthrene-Induced Mammary Tumors*

Tissue	Mouse with mammary tumors	Normal mice*
	μc. per mole C	*μc. per mole C*
1. Blood	4.18	0.25, 0.16, 0.10
2. " (vacuum-dried 24 hrs. over H_2SO_4)	0.13	
3. Mammary tumor	4.03	
4. " " (vacuum-treated)	0.88	
5. Control mammary tissue..	0.88	

* See Bryan *et al.* (1), p. 944.

urethane in the blood and tissues of a mouse with methylcholanthrene-
induced tumors and the retention in normal mice.

DISCUSSION

From a consideration of the data given in Table V and in a previous
paper (1), it is apparent that the tissues of the neoplastic mice were higher
in radioactivity than were normal control mouse tissues at 24 hours after
injection of radioactive urethane. In the case of whole blood, 97 per cent
of this activity was volatile *in vacuo*, as shown in Table V. We have shown
that free urethane may be removed from tissue by vacuum treatment.

The chemical analyses for urethane, Table IV, show that blood of animals
with advanced spontaneous mammary carcinoma averaged 21 per cent
higher in urethane than did normal mice at the end of 24 hours. The
urethane in these four groups of carcinomatous animals ranged from 0.17
to 0.37 mg. per 0.5 ml. of blood 24 hours after injection.

It is significant to compare the amount of urethane found by chemical analysis and that determined by radioactive measurements. Only 44.2 per cent of the total radioactivity from carbonyl-labeled urethane injected into the mouse with methylcholanthrene-induced mammary tumors was recovered in the expired carbon dioxide in the 24 hour collection period (1). It has been observed that greater than 90 per cent of the carbonyl carbon from urethane is exhaled as CO_2 within a similar period in the case of normal mice. About 11 per cent of the total activity was found in the 24 hour urine sample of the carcinomatous mice which is of the same order as that observed in normal mice. About 45 per cent of the total activity was, therefore, distributed throughout the body of the animal with methylcholanthrene-induced tumors. This may be compared with the retention of radioactive carbon at the end of a 24 hour period by normal mice which we have found to be of the order of 0.6 to 2 per cent of the total amount injected.

Based upon 23 gm. as the weight of the carcinomatous mouse used, it is calculated that about 1.47 gm., 6.4 per cent, represent the weight of blood. The total amount of the radioactive carbon remaining in the mouse at 24 hours corresponds to 15.4 mg. of urethane, or 44 per cent of the 35 mg. of urethane injected. Therefore, assuming equal distribution, the blood contained approximately 0.98 mg. of urethane or about 0.33 mg. per 0.5 ml. of blood. It is obvious from Table IV that this amount is included in the range of values found by chemical determination and that most of the excess radioactivity over that of normal animals can thus be accounted for as urethane. It is, therefore, assumed that the differences in retention of radioactive carbon by normal mice and those with advanced spontaneous mammary carcinomas (1) are due principally to a failure of these neoplastic mice to catabolize the urethane molecule at the normal rate and are not due to retention of some metabolite containing the carbonyl carbon of the urethane molecule.

From a consideration of the amount of radioactivity exhaled as carbon dioxide in a 24 hour period, together with that excreted in urine, it is possible to calculate that the amount remaining in the animals with spontaneous lymphoid leucemia (1) at the end of 24 hours corresponds to 0.06 mg. of urethane in one case and 0.10 mg. in another. These values are in the range in which the chemical determination cannot be considered very accurate; however, 0.05 mg. of free urethane was found by this method in animals with spontaneous lymphoid leucemia.

SUMMARY

1. Chemical analyses of blood from normal mice have shown that the urethane is practically all eliminated from the blood of normal mice in

24 hours after injection of 1400 mg. per kilo, but the blood of mice with advanced spontaneous mammary carcinoma contains about 21 per cent more urethane than blood of the normal animals.

2. The amount of urethane calculated from radioactive assays to be present in the blood of a mouse with advanced methylcholanthrene-induced mammary carcinomas at 24 hours corresponds closely with that determined chemically in the blood of a group of mice with advanced spontaneous mammary carcinoma. It is concluded, therefore, that the excess radioactivity present in the neoplastic animals is due principally to free urethane and results from a lower rate of catabolism of the urethane in these animals.

BIBLIOGRAPHY

1. Bryan, C. E., Skipper, H. E., and White, L., Jr., *J. Biol. Chem.*, **177,** 941 (1949).
2. Winnick, T., *Ind. and Eng. Chem., Anal. Ed.*, **14,** 523 (1942).
3. Skipper, H. E., Bryan, C. E., White, L., Jr., and Hutchison, O. S., *J. Biol Chem.*, **173,** 371 (1948).

THE GASOMETRIC DETERMINATION OF α-AMINO ACIDS BY THE *peri*-NAPHTHINDAN-2,3,4-TRIONE HYDRATE-CARBON DIOXIDE METHOD IN PURE SOLUTIONS, WITH REMARKS UPON ITS USE IN BLOOD AND URINE

By RADWAN MOUBASHER and ALY SINA

(From the Faculty of Science, Fouad Ist University, Cairo, Egypt)

Oxidation of α-amino acids by ninhydrin (I) leads to the formation of carbon dioxide and diketohydrindylidenediketohydrindamine (II) together with an aldehyde, as noted by Ruhemann (1) and by Grassmann and von Arnim (2).

(I)

(II)
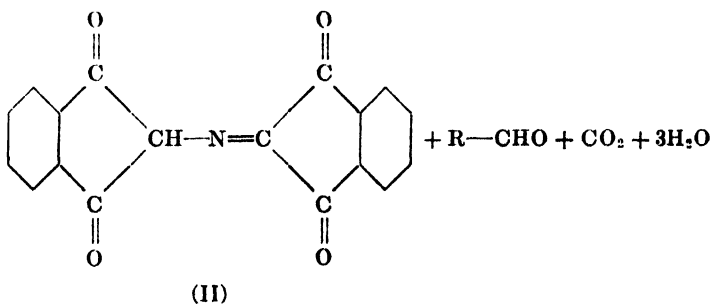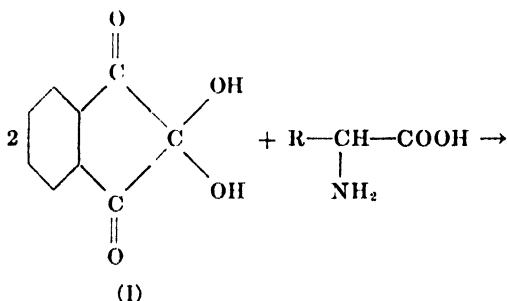
Van Slyke and Dillon (3) and Mason (4) found that the carboxyl group is thereby split off quantitatively. On the basis of this finding they developed a manometric method for the quantitative determination of the carboxyl group in amino acids.

Moubasher (5) has used *peri*-naphthindan-2,3,4-trione hydrate (III) as a new reagent for the quantitative determination of α-amino acids through the formed aldehydes, as this reagent is able to decompose quan-

titatively the α-amino acids in acidified boiling water to the next lower aldehyde with 1 carbon atom less, together with ammonia, carbon dioxide, and dihydroxyketo-*peri*-naphthindene (IV).
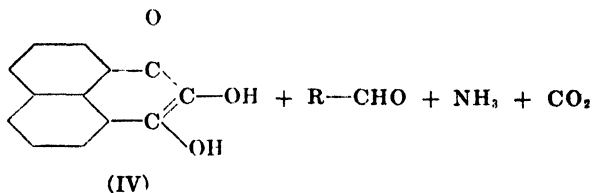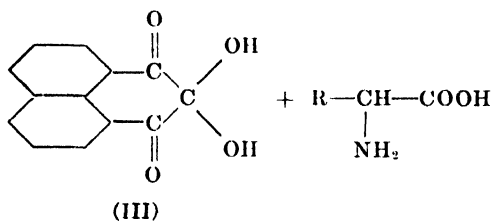
In the present work we have found that α-amino acids, when treated with an excess of *peri*-naphthindan-2,3,4-trione hydrate, evolve quantitatively the carbon dioxide of their carboxyl groups.
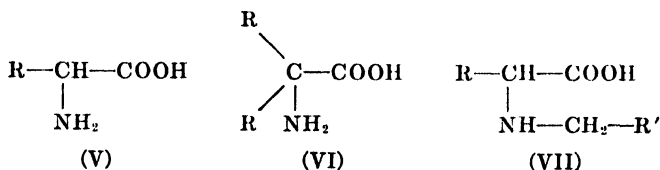
Specificity—(V) and (VI) are molecular structures which provide carboxyl groups yielding carbon dioxide quantitatively by reaction with *peri*-naphthindan-2,3,4-trione hydrate. Structure (V) represents amino acids with primary —NH_2 groups. Structure (VI) represents amino acids in which the hydrogen atom attached to the α-carbon atom is substituted. Thus, aminoisobutyric acid reacts with *peri*-naphthindan-2,3,4-trione hydrate (III), giving acetone, carbon dioxide, and ammonia (*cf.* Schönberg, Moubasher, and Mostafa (6)). Structure (VII) represents amino acids with secondary —NH groups, *e.g.* sarcosine, which decomposes with evolution of carbon dioxide. On the other hand amino
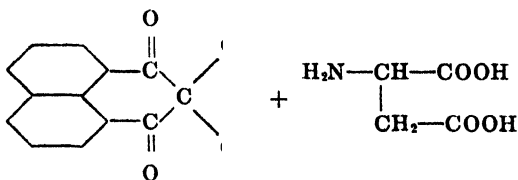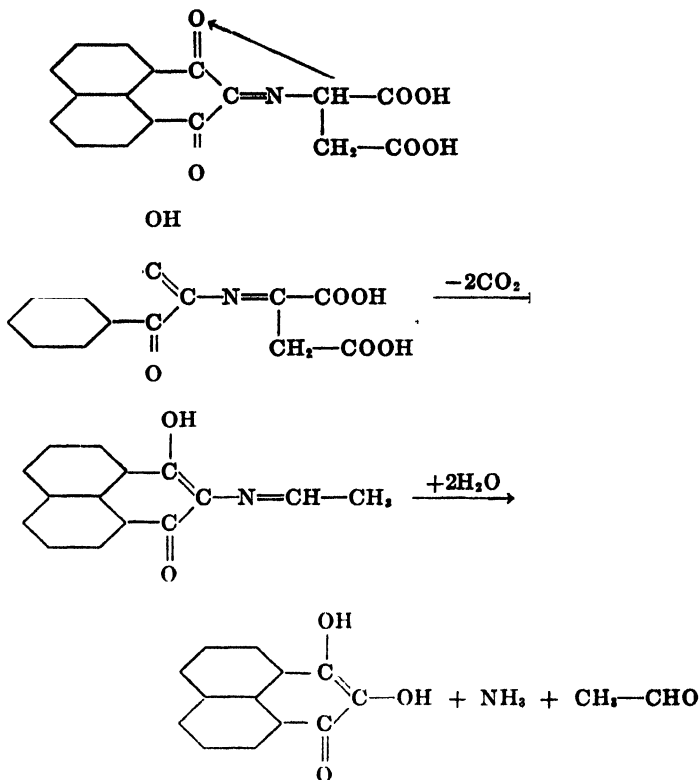
acids in which the carboxyl groups are either esterified or in the form of their amides do not react. This behavior is in good agreement with that found with ninhydrin and its analogues (cf. Van Slyke et al. (7)). If the amino group is moved from the α to the β or γ position, the reactivity of the carbonyl group diminishes but may not disappear (cf. Moubasher and Awad (8)).

We have also found that aspartic acid, when treated with peri-naphthindan-2,3,4-trione hydrate, evolves quantitatively the carbon dioxide of both its carboxyls, with the final formation of acetaldehyde (cf. Moubasher (5)). For this reaction the accompanying scheme is advanced (cf. (1)).

In contrast to the action of ninhydrin on glutamic acid, which causes the evolution of carbon dioxide from one carboxyl group (cf. Van Slyke et al. (7)), peri-naphthindan-2,3,4-trione hydrate causes the evolution of carbon dioxide from both carboxyls.

Remarks on Use of peri-Naphthindan-2,3,4-trione Hydrate in Gasometric Determination of α-Amino Acids in Blood and Urine—When heated

with boiling water at pH 4.7, urea by itself slowly gives off carbon dioxide (0.01 mole in 5 minutes), but excess of *peri*-naphthindan-2,3,4-trione hydrate retards this decomposition (*cf.* Errera and Sorgés (9)) owing to the formation of
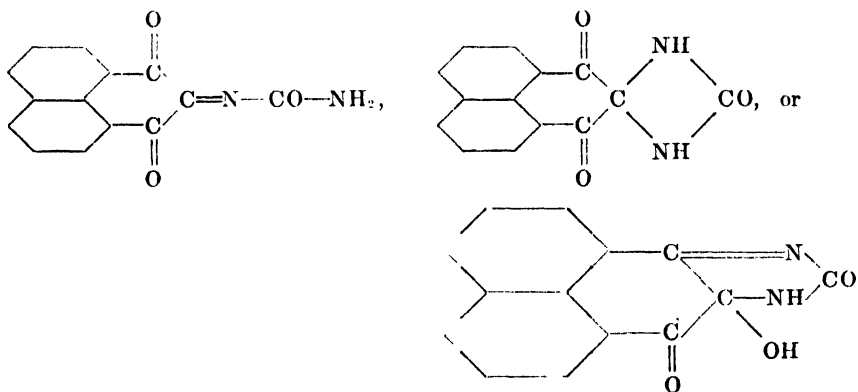
These ureides are similar in stability and formation to their analogues of ninhydrin. The application of this reaction in blood and urine has been previously mentioned with use of ninhydrin (*cf.* Van Slyke *et al.* (10, 11)). Accordingly, for blood analysis, there is no need to take any special precaution on this account. When working with samples of urine, the urea-amino acid ratio will be so high that it is preferable to remove the urea by means of a specially prepared amino acid-free urease. Keto acids, *e.g.* pyruvic and acetoacetic acids, evolve carbon dioxide under the conditions of the determination of carbon dioxide from amino acid carboxyl groups. Also, ascorbic acid evolves about 0.1 mole of carbon dioxide in the time required for complete reaction with *peri*-naphthindan-2,3,4-trione hydrate (*cf.* Moubasher (12)). The concentration of ascorbic acid in body fluids and excretions is too slight, however, to affect significantly the amino acid determinations. In spite of the fact that simple organic acids such as acetic and hydroxy acids do not yield carbon dioxide under the experimental conditions described, this method cannot be applied to blood and urine filtrates, because traces of these volatile acids distil with the carbon dioxide and cause positive errors, and, if picric acid filtrates are used, some of the picric acid also distils over and causes error.

EXPERIMENTAL

This method is essentially based on that used by Van Slyke *et al.* (7) in the determination of α-amino acids through the evolved carbon dioxide by the action of ninhydrin.

Reagents—

·1. Choice of pH for conducting the reaction. It is necessary to control the pH at which the reaction with *peri*-naphthindan-2,3,4-trione hydrate is carried out. It has been found that buffers of pH 2.5 and 4.7 are the most suitable and yield excellent results (*cf.* (5)). In the present work we have used a buffer of pH 4.7, which is prepared by grinding and intimately mixing 17.65 gm. of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) and 8.4 gm. of citric acid ($C_6H_8O_7 \cdot H_2O$) (*cf.* (13)).

2. *peri*-Naphthindan-2,3,4-trione hydrate freshly crystallized and pulverized.

3. Approximately 0.125 N $Ba(OH)_2$ solution· prepared by mixing 5 volumes of 0.15 N $Ba(OH)_2$ with 1 volume of 12 per cent $BaCl_2$ solution.

4. A standard solution of N/35 HCl.

5. 1 per cent solution of phenolphthalein in 96 per cent alcohol to be used as an indicator.

6. 10 per cent solution of sodium hydroxide in water for use in the reservoir of carbon dioxide-free air.

7. Two 5 cc. micro burettes, one for the $Ba(OH)_2$ solution and the other for the standard HCl solution.

Apparatus—The reaction is carried out in a relatively simple apparatus similar to that used by Van Slyke (*cf.* (7)) with some modifications. It consists of two Erlenmeyer flasks (*A* and *B*) made of Pyrex glass, each having a capacity of 20 cc., which are attached to the limbs by means of ground joints. The limbs, which are made also of Pyrex glass, are bent as shown in Fig. 1. At the top is attached a tap (*C*).

A reservoir of carbon dioxide-free air.

Calibrated micro tubes to hold 40 mg. of buffer and 15 and 30 mg. of *peri*-naphthindan-2,3,4-trione hydrate.

Procedure

In flask *B* are placed 2 cc. of the amino acid solution and then a calibrated micro tube containing 40 mg. of buffer of pH 4.7. Through flask *A* is now passed a stream of carbon dioxide-free air (about 500 cc.) and then, by means of a micro burette fitted with a soda lime tube, 1 cc. of $Ba(OH)_2$ solution is added. In the amino acid flask *B* is now dropped the calibrated micro tube containing the proper amount of *peri*-naphthindan-2,3,4-trione hydrate. Both flasks are quickly attached to the limbs of the apparatus and evacuated through tap *C* with a high vacuum pump. Tap *C* is closed and the apparatus is dipped in a boiling water bath up to about three-fourths its height, where it is left for the proper time with occasional shaking. After a specified period, the limb attached to the barium hydroxide flask is taken out of the boiling bath, flask *A* is im-

mersed in ice-cold water, and the distillation of the contents of flask *B* is continued until the majority of the liquid has passed over. This takes 2 to 3 minutes. The apparatus is now cooled and then CO_2-free air is allowed to enter through tap *C*. Flask *A* is detached, 1 drop of phenolphthalein is added, and it is immediately titrated against N/35 HCl.

A blank experiment is performed in which an equal volume of distilled water is used instead of the amino acid solution and with all the reagent₅
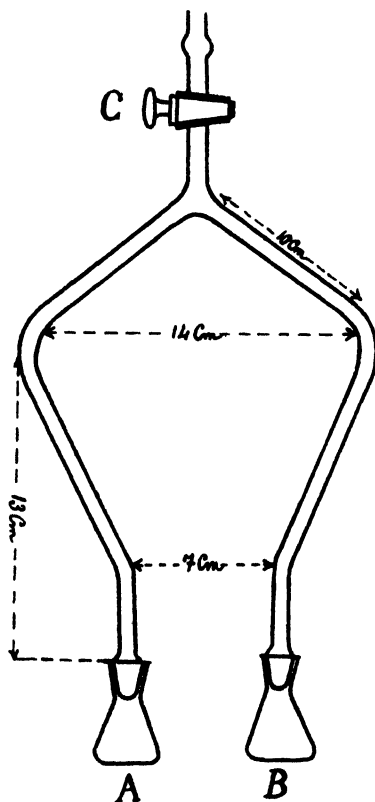
Fig. 1. Apparatus for gasometric determination of amino acids

used except the *peri*-naphthindan-2,3,4-trione hydrate, as the latter does not evolve carbon dioxide under the experimental conditions described.

The following amino acids are estimated: glycine, alanine, valine, leucine, phenylglycine, aspartic acid, and glutamic acid. From Table I, it is evident that for 100 mg. of the amino acid used the error is about ±4 per cent.

We did not find it necessary to use any caprylic alcohol or alundum to prevent foaming or bumping, as used in the Van Slyke procedure. It seems that the precipitated dihydroxynaphthindene together with the micro tubes helps to avoid this difficulty, and, even if bumping occurs in rare cases, the bent limb of the apparatus prevents the liquid from passing over to the baryta flask. Ground joint flasks offer greater security against loss of carbon dioxide and avoid the difficulties arising from the use of rubber in the Van Slyke apparatus.

Action of peri-Naphthindan-2,3,4-trione Hydrate on Sarcosine—By the same procedure, the decomposition of sarcosine with *peri*-naphthindan-2,3,4-trione hydrate was tried and the evolution of carbon dioxide was verified, but the reaction was not found to be quantitative.

The use of *peri*-naphthindan-2,3,4-trione hydrate has the advantage of being cheaper than ninhydrin and can be recovered from the above

TABLE I

Results of Amino Acid Determination

Amino acid	Amount	Time of heating	Buffer	N/35 HCl	Recovery
	mg.	*min.*	*mg.*	*cc.*	*per cent*
Glycine....	1	8	20	1.0	102
Alanine....	2	15	40	1.5	95.5
Valine..	2	15	40	1.2	100
Leucine........	1	6	20	0.52	101
Phenylglycine	1	8	20	1.02	106
Aspartic	2	15	40	2.02	96
Glutamic......	1	8	20	1.02	107

experiment by treating the contents of the amino acid flask with bromine water (*cf.* (5)).

SUMMARY

1. *peri*-Naphthindan-2,3,4-trione hydrate decomposes α-amino acids with the quantitative evolution of carbon dioxide. The loss of the reagent is small, as it can be regenerated after the experiment.

2. Both aspartic and glutamic acids react with *peri*-naphthindan-2,3,4-trione hydrate with the quantitative evolution of 2 moles of carbon dioxide.

3. This method cannot be applied to blood and urine filtrates.

BIBLIOGRAPHY

1. Ruhemann, S., *J. Chem. Soc.*, **99**, 792, 1486 (1911).
2. Grassmann, W., and von Arnim, K., *Ann. Chem.*, **509**, 288 (1934).

3. Van Slyke, D. D., and Dillon, R. T., *Proc. Soc. Exp. Biol. and Med.*, **34**, 362 (1936); *Compt-rend. trav. Lab. Carlsberg*, **22**, 480 (1938).

4. Mason, M. F., *Proc. Soc. Exp. Biol. and Med.*, **37**, 111 (1937); *Biochem. J.*, **32**, 719 (1938).

5. Moubasher, R., *J. Biol. Chem.*, **175**, 187 (1948).

6. Schönberg, A., Moubasher, R., and Mostafa, A., *J. Chem. Soc.*, 176 (1948).

7. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 671 (1941).

8. Moubasher, R., and Awad, W., *J. Chem. Soc.*, in press.

9. Errera, G., and Sorgés, F., *Gazz. chim. ital.*, **43**, 625 (1914); *Chem. Abstr.*, **8**, 1274 (1914).

10. Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, **150**, 231 (1943).

11. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, **150**, 251 (1943).

12. Moubasher, R., *J. Biol. Chem.*, **176**, 529 (1948).

13. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).

RELATION OF DIETARY FOLIC ACID AND VITAMIN B$_{12}$ TO ENZYME ACTIVITY IN THE CHICK*

BY J. N. WILLIAMS, JR., C. A. NICHOL, AND C. A. ELVEHJEM

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

The relationship of folic acid (PGA) to certain enzyme systems has recently been the subject of investigation by workers in several laboratories. In 1948 Kalckar and Klenow (1–3) demonstrated the inhibitory effect of folic acid upon milk xanthopterin oxidase *in vitro*. Subsequently Keith *et al.* (4), studying the effect of dietary PGA upon enzymes, observed that activity of xanthine oxidase in chick liver was strongly depressed as the concentration of folic acid was increased in the ration. Quite recently Kelley (5) observed that rat liver D-amino acid oxidase activity was decreased to some extent by feeding PGA.

Although the relationship of folic acid and vitamin B$_{12}$ to hemoglobin production is quite well known (6), some of the basic metabolic functions of these two vitamins, particularly of vitamin B$_{12}$, are at present obscure.

In view of this information the effects of variations in dietary intake of vitamin B$_{12}$ and folic acid on the following enzyme systems were investigated: xanthine oxidase, D-amino acid oxidase, endogenous respiration, and catalase. Since we have experimental evidence[1] that folic acid strongly inhibits endogenous respiration of rat liver *in vitro*, the effects of dietary folic acid and vitamin B$_{12}$ upon endogenous oxidative activity have been studied. The influence of the two vitamins in question upon liver catalase activity has been investigated for several reasons. First, the porphyrin group is common to both catalase and hemoglobin; second, catalase destroys metabolic hydrogen peroxide, which is produced by oxidation of D-amino acids and xanthine; and third, metabolic hydrogen peroxide is believed to be involved in the destruction of hemoglobin (7).

As a preliminary investigation into the metabolic disorder producing paralytic symptoms in chicks on a folic acid-deficient diet containing

vitamin B_{12} (6), blood glucose determinations were made on the various groups of experimental animals.

Straight run (New Hampshire males $\times$ single comb white Leghorn females) cross-bred chicks which were the offspring of hens fed Diet B-1 (8) were used as experimental animals. The chicks were housed in electrically heated batteries with raised screen floors and were fed and watered *ad libitum*. Weights and hemoglobin values of the chicks were recorded just prior to use in the enzyme studies. Blood samples for hemoglobin and glucose determinations were taken by direct puncture of a wing vein.

Throughout the feeding period all chicks received a folic acid-deficient ration containing sucrose 61, alcohol-extracted casein 18, gelatin 10, Salts V (9) 6, soy bean oil 5, L-cystine 0.3, and fortified haliver oil (60,000 U. S. P. units of vitamin A, 6000 A. O. A. C. units of vitamin D_3 per gm.) 0.4 per cent; thiamine hydrochloride 0.3, riboflavin 0.6, nicotinic acid 5.0, pyridoxine hydrochloride 0.4, calcium pantothenate 2.0, biotin 0.02, choline chloride 150, inositol 100, 2-methyl-1,4-naphthoquinone 0.05, and α-tocopherol 0.3 mg. per cent.

Four groups of chicks were maintained on the synthetic ration for 4 weeks before being used in the enzyme studies. When supplemented with folic acid or vitamin B_{12}, the chicks received 200 γ of folic acid[2] per 100 gm. of ration and 0.7 per cent charcoal-vitamin B_{12} concentrate[2] (equivalent to approximately 3 γ of vitamin B_{12} per 100 gm. of ration). Some of the chicks were placed on the basal ration for 2 weeks before supplementation with PGA or vitamin B_{12}. The remainder received the supplements throughout the 4 week period. Each of the four groups of chicks received the following supplements to the basal ration, respectively: Group I, folic acid plus vitamin B_{12}; Group II, folic acid alone; Group III, vitamin B_{12} alone; and Group IV no supplement. In addition to these four groups of chicks an additional group was maintained on a good stock ration for 4 weeks.

When used in the enzyme experiments, the animals were decapitated and bled. The livers were removed immediately, chilled in cracked ice, blotted free of moisture, and weighed. A portion of each liver was homogenized for 3 minutes in a Potter-Elvehjem homogenizer with 5 volumes of ice-cold 0.039 M sodium potassium phosphate buffer (10) and strained through gauze.

Xanthine oxidase activity of the homogenates was determined by a method outlined by Keith *et al.* (4). D-Amino acid oxidase was measured by the method of Axelrod, Sober, and Elvehjem (11), and catalase by the method of Jolles (12). The first 10 minute oxygen uptake before the substrate was added in the D-amino acid oxidase determinations was taken as a measure of endogenous respiration of the livers.

Liver nitrogen and dry weight determinations were made in most cases. Whenever possible, enzyme activity, blood glucose, nitrogen, and dry weight determinations were made on the same animals.

TABLE I

Relationship of Vitamin Supplements to Enzyme Activity

	No. of chicks	Ration supplement		Liver enzyme activity
D-Amino acid oxidase	13	PGA, vitamin B_{12}	97	Units = μl. O_2 uptake per
	6	"	100	hr. per gm. liver at 30°
	13	Vitamin B_{12}	700	
	15	None	395	
	6	(Stock ration)	230	
Xanthine oxidase	13	PGA, vitamin B_{12}	2.0	Units = mg. uric acid
	6	"	1.6	formed per 1½ hrs. per
	13	Vitamin B_{12}	4.6	gm. liver at 25°
	12	None	5.3	
	6	(Stock ration)	2.4	
Endogenous respiration	13	PGA, vitamin B_{12}	2490	Units = μl. O_2 uptake per
	6	"	2500	hr. per gm. liver at 30°
	13	Vitamin B_{12}	2310	
	15	None	2410	
	6	(Stock ration)	1880	
Catalase	13	PGA, vitamin B_{12}	94	Units = monomolecular
	6	"	97	reaction constant at
	13	Vitamin B_{12}	103	0° × dilution of liver
	11	None	107	
	6	(Stock ration)	166	

RESULTS AND DISCUSSION

The results of the enzyme determinations are presented in Table I. Enzyme activities for the two lots of chicks given supplements of the vitamins in question throughout the 4 week feeding period or for only the last 2 weeks have been averaged together, since both lots gave essentially the same results.

Calculation of the enzyme activities in terms of wet weight, nitrogen, or dry weight of the liver gives essentially the same picture. For this reason we have reported the results upon a wet weight basis only.

When both PGA and vitamin B_{12} are absent from the diet, activity of

D-amino acid oxidase in the liver is about 4 times greater than that observed when PGA is present. However, if vitamin B_{12} is present without PGA in the diet, a 7-fold increase in D-amino acid oxidase activity is observed. It appears, therefore, that vitamin B_{12} and folic acid have dissimilar effects upon this enzyme. When folic acid is included in the diet, however, the stimulatory effect of vitamin B_{12} disappears.

In contrast to the results with D-amino acid oxidase, xanthine oxidase activity is not increased by the level of vitamin B_{12} fed, even if PGA is omitted from the ration. In fact, vitamin B_{12} appears to depress activity of this enzyme slightly when PGA is absent from the diet. Since both D-amino acid oxidase and xanthine oxidase are flavin enzymes, the apparent inhibition of activity by folic acid can possibly be explained by a direct action of the vitamin upon the enzyme protein or prosthetic group. This action is perhaps competitive in nature in view of the structural similarities of the pterin nucleus both to the purines and to the flavin group. However, the fact that D-amino acid oxidase activity is higher when vitamin B_{12} is included in the diet (in the absence of PGA) is perhaps explainable by an increased synthesis of enzyme protein rather than a direct stimulation of the enzyme already present. This point remains to be checked by direct *in vitro* studies with vitamin B_{12}, however.

From the results in Table I, it appears that dietary PGA at the levels employed has only a negligible influence upon endogenous respiration. These results are contrary to those obtained when PGA is added directly to a flask containing rat liver homogenate.[1] It is possible that the effects of the relatively low dietary levels upon endogenous oxygen consumption are obscured by dilution of the vitamin in the tissues of the intact animal. It is also conceivable that the depressant effects of folic acid occur mainly in relation to specific flavin enzymes. Consequently, if most of the endogenous respiration in chick liver is due to oxidation of metabolites not involving the flavin enzymes in question, the depressant effect of PGA upon endogenous oxygen consumption may not be easily apparent.

From the results in Table I, it appears that catalase activity is independent of either dietary PGA or vitamin B_{12}. It was thought that perhaps the activity of catalase would increase when a greater need for it appeared, as in a folic acid deficiency. On the other hand, there is certainly no decrease in catalase activity when PGA is absent from the diet. It would appear, therefore, that the decrease in blood hemoglobin concomitant with a folic acid deficiency is due to an effect other than inadequate porphyrin synthesis.

The results of the enzyme determinations for the group of stock chicks are somewhat different in endogenous respiration and catalase activity from the results of the chicks on synthetic rations, although all of the animals employed were comparable in age and heredity. The reasons

for these differences are not clear, although they possibly reflect a healthier status of the animals on stock ration because of the presence of unknown dietary factors.

Values for average chick weights, hemoglobin, ratios of liver to body weight, and blood glucose for the different groups of chicks on synthetic ration are reported in Table II. The results from those animals receiving supplements of PGA or vitamin B_{12} throughout the 4 week period are presented, since blood glucose was determined only upon these chicks. It appears that, although vitamin B_{12} alone exerts little influence upon hemoglobin formation, it has a strong effect in increasing the body weight of the chicks. Folic acid alone has a similar but greater effect upon chick weight. Under our experimental conditions weight response is optimal only when both vitamins are given.

From the work of Keith et al. (4) it appears that dietary folic acid is inversely related to the ratio of liver to body weight. Our results indicate

TABLE II

Effect of Dietary Folic Acid and Vitamin B_{12} on Weight and Blood Constituents in Chicks

Ration supplement*	Average weight of chicks	Hemoglobin	Ratio of liver to body weight	Blood glucose
	gm.	*per cent*		*mg. per cent*
Folic acid, vitamin B_{12}	309	9.1	0.028	181
" "	263	8.6	0.029	180
Vitamin B_{12}..	146	5.2	0.036	246
None.. ..	90	6.8	0.036	238

* Six chicks in each group.

this to be the case, and that vitamin B_{12}, even in the absence of PGA, has little influence upon this ratio.

The results of blood glucose determinations indicate that in the absence of dietary folic acid blood sugar concentration is increased. Dietary vitamin B_{12}, however, at the levels employed appears to have little effect.

The apparently synergistic action of folic acid and vitamin B_{12} upon weight and hemoglobin of the chick is not clearly indicated among the metabolic factors we have studied in this paper. In only one case, D-amino acid oxidase, do we find a positive effect of vitamin B_{12} upon enzyme activity, and this effect is unlike that of folic acid.

SUMMARY

Studies on the influence of dietary folic acid and vitamin B_{12} on D-amino acid oxidase, xanthine oxidase, catalase, and endogenous respiration of chick liver have been presented.

Reports that dietary folic acid is related inversely to the two flavin enzymes have been confirmed. It appears that catalase and endogenous respiration are not directly controlled by either vitamin B_{12} or PGA.

Chicks fed a diet supplemented with vitamin B_{12} without folic acid show higher D-amino acid oxidase activity than those not receiving vitamin B_{12}.

Vitamin B_{12} augments the effect of PGA in weight responses.

Dietary folic acid is inversely related to blood glucose, while under the conditions of our experiments vitamin B_{12} appears to have little direct relationship to this blood component.

BIBLIOGRAPHY

1. Kalckar, H. M., and Klenow, H., *J. Biol. Chem.*, **172,** 349 (1948).
2. Kalckar, H. M , and Klenow, H., *J. Biol. Chem.*, **172,** 351 (1948).
3. Kalckar, H. M., Kjeldgaard, N. O., and Klenow, H., *J. Biol. Chem.*, **174,** 771 (1948).
4. Keith, C. K., Broach, W. J., Warren, D., Day, P. L., and Totter, J. R., *J. Biol. Chem.*, **176,** 1095 (1948).
5. Kelley, B., *Federation Proc.*, **8,** 212 (1949).
6. Nichol, C. A., Harper, A. E., Dietrich, L. S., and Elvehjem, C. A., *Federation Proc.*, **8,** 233 (1949).
7. Granick, S., and Gilder, H., *Advances in Enzymol.*, **7,** 305 (1947).
8. Robblee, A. R., Nichol, C. A., Cravens, W. W., Elvehjem, C. A., and Halpin, J. C., *Poultry Sc.*, **27,** 442 (1948).
9. Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **148,** 163 (1943).
10. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **140,** 725 (1941).
11. Axelrod, A. E., Sober, H. A., and Elvehjem, C. A., *J. Biol. Chem.*, **134,** 749 (1940).
12. Jolles, A., *Münch. med. Wochschr.*, **51,** 2083 (1904).

MICROBIOLOGICAL DETERMINATION OF ISOLEUCINE IN PROTEINS AND FOODS

By MILLARD J. HORN, D. BREESE JONES, AND AMOS E. BLUM

(*From the Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, United States Department of Agriculture, Washington*)

Streptococcus zymogenes (1), *Streptococcus lactis* (2), *Lactobacillus fermenti* 36 (3), *Lactobacillus arabinosus* (4–11), *Leuconostoc mesenteroides* P-60 (12), and *Streptococcus faecalis* (13–16) have all been used or recommended for the assay of isoleucine.
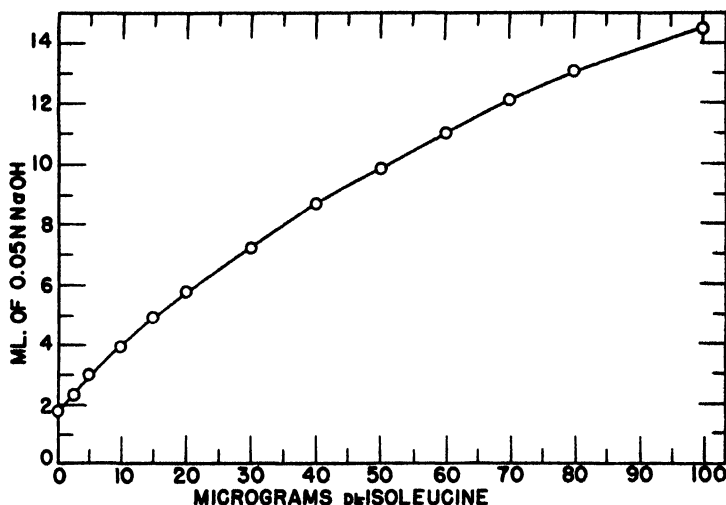
FIG. 1. Standard curve for isoleucine with *Leuconostoc mesenteroides*

The discovery by Smith and Greene (17) of an inactive isomer, alloisoleucine, in the synthetic isoleucine preparations has focused attention on the purity of preparations used to develop the standard curves. It was found that most of the commercial samples of isoleucine were not pure enough to use in preparing the standard curve, one sample assaying only about 28 per cent of isoleucine. However, one of these preparations was found to contain no alloisoleucine and was used as the standard. In order to check the results obtained with *L. mesenteroides*, a second organism *S. faecalis* was used. The values obtained were in close agreement.

TABLE I

Differences in Acid Production by Leuconostoc mesenteroides in Media with Various Isoleucine Preparations

L-Isoleucine	L Sample 1	L Sample 2	DL Sample 3	DL Sample 4	DL Sample 5
γ	ml.	ml.	ml.	ml.	ml.
5	2.9	3.4	3.3	3.9	3.4
10	3.9	5.2	4.8	5.8	5.2
15	4.8	6.4	6.1	7.2	6.7
20	5.8	7.6	7.1	8.7	8.0
25	6.5	8.5	8.1	9.9	9.2
30	7.3	9.5	9.0	11.0	10.1
40	8.7	11.1	10.7	13.1	12.0
50	9.8	12.9	12.1	14.5	13.7

The results are expressed in ml. of 0.05 N sodium hydroxide.

TABLE II

Recovery of Isoleucine Added to Protein Hydrolysates

Protein hydrolysate	Isoleucine				
	In hydrolysate*	Added	Total	Found	Recovery
	γ	γ	γ	γ	per cent
Arachin	4.25	5.00	9.25	9.40	102
"	8.50	5.00	13.50	14.00	103
"	12.75	5.00	17.75	17.85	101
Casein	10.70	2.50	13.20	13.50	102
"	10.70	5.00	15.70	15.75	101
" .	10.70	7.50	18.20	18.25	101
"	10.70	10.00	20.70	20.75	101
Corn, whole, yellow	5.25	5.00	10.25	10.25	100
" " " . . .	5.25	10.00	15.25	15.75	103
" " " .	5.25	20.00	25.25	25.00	99
" " "	5.25	30.00	35.25	34.75	98
Milk, dry, skim	8.50	2.50	11.00	11.10	101
" " " .	8.50	5.00	13.50	13.50	100
" " "	8.50	7.50	16.00	16.50	103
" " "	8.50	10.00	18.50	18.50	100

* Uncorrected for moisture and ash.

EXPERIMENTAL

Leuconostoc mesenteroides P-60 was employed in the assays described.[1]

Basal Medium—The basal medium was the same as that used in the assay of phenylalanine (18).

[1] Obtained from the American Type Culture Collection, Georgetown University School of Medicine, Washington, D. C.

Assay Procedure—The procedures followed for the cultures, inoculum, and preparation of samples were identical with those described in other papers (19, 20).

TABLE III

Isoleucine Content of Some Proteins and Foods Determined at Different Assay Levels*

Assay level	Lactalbumin		Phaseolin		Brazil nut meal		Whole dried egg	
γ	γ	per cent	γ	per cent	γ	per cent	γ	per cent
100	5.80	5.80	6.00	6.00				
200	11.50	5.75	11.80	5.90	3.50	1.75	8.00	4.00
300	17.25	5.75	18.00	6.00	.			
400	22.75	5.70	24.00	6.00	7.10	1.78	15.60	3.90
500								
600					10.50	1.75	24.00	4.00
800					13.50	1.69	32.15	4.02
1000								
1500								
2000								
3000								
4000								
Average ..		5.75		5.98		1.74		3.98

Assay level	Whole yellow corn		Oatmeal		White rice		Whole rye	
γ	γ	per cent	γ	per cent	γ	per cent	γ	per cent
100								
200								
300								
400								
500	2.45	0 49	3.40	0.68				
600								
800								
1000	5.00	0.50	6.75	0.68	3.10	0.31	4.50	0.45
1500	7.15	0.48	10.25	0.68				
2000	9.60	0.48	13.50	0.68	6.30	0.31	9.25	0.46
3000					9.00	0.30	13.40	0.45
4000					12.00	0.30	18.00	0.45
Average. .		0.49		0.68		0.31		0.45

* Uncorrected for moisture and ash.

Preparation of Isoleucine Standards—In Table I there is presented a comparison of acid production obtained by assaying five different isoleucine samples purchased from four different commercial manufacturers. It is apparent that there is quite a difference in the purity of these compounds. Assuming 50 per cent activity for the DL compounds, the increase in acidity per unit of L-isoleucine indicates that Sample 4 is the purest

compound. This sample gave no change in acid production after repeated recrystallization from dilute alcohol and was considered sufficiently pure

TABLE IV

Reproducibility of Isoleucine Content by Separate Assays*

Material	Assay 1	Assay 2	Assay 3	Average
	per cent	*per cent*	*per cent*	*per cent*
Arachin	4.19	4.16		4.18
Barley, pearled	0.48	0.48		0.48
Casein..	5.31	5.14	5.21	5.22
Conarachin	3.76	3.69	3.74	3.73
Corn germ, defatted	0.79	0.73		0.76
Corn, whole, yellow	0.49	0.52		0.51
Cottonseed globulin	4.04	4.19		4.12
Edestin .	5.22	5.11	5.17	5.17
Gelatin (Bacto)	1.01	1.09		1.05
Milk, dry, skim	2.21	2.13		2.17
Ovalbumin (crystalline)	6.90	7.00		6.95
Phaseolin (navy bean)	6.06	5.98		6.02
Wheat bran globulin	3.54	3.68		3.61
Zein...	4.89	4.70	4.77	4.79

* Uncorrected for moisture and ash.
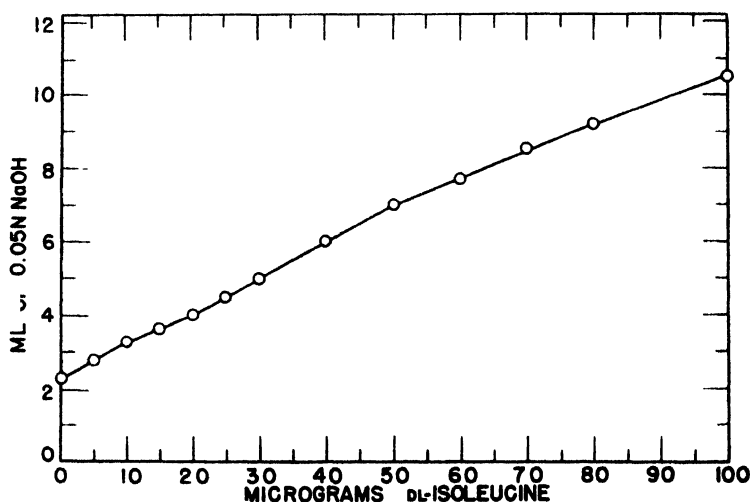
FIG. 2. Standard curve for isoleucine with *Streptococcus faecalis*

to use in preparing the standard curve (Fig. 1). The titration values on this curve were not altered by the addition to the medium of 1.2 mg. of any of the other nineteen amino acids.

TABLE V

Isoleucine Content of Some Proteins and Foods with Use of Two Different Organisms

Percentages calculated for ash- and moisture-free material.

Material	N	Isoleucine		Values from literature
		L. mesenteroides	*S. faecalis*	
	per cent	per cent	per cent	
Arachin..... ..	18.30	4.55	4.38	
Casein..	16.07	5.63	5.82	6.4 (8), 6.0 (9), 5.6 (13), 8.0 (14), 7.6 (16), 5.2 (21)
Coconut globulin. ..	17.42	4.43	4.49	.
Conarachin........ ..	18.20	3.98	4.02	
Cottonseed globulin. . .	18.00	4.62	4.52	2.5 (22), 2.2 (23)
Edestin.........	18.55	5.54	5.46	6.47 (16), 4.7 (17)
Gelatin (Bacto)....	18.32	1.25	1.27	1.5 (9), 1.72 (13), 1.9 (14), 1.7 (23)
Glycinin......	17.30	5.69	5.84	
Lactalbumin....	15.39	6.12	5.89	6.0 (9)
Ovalbumin (crystalline).......	15.98	7.41	7.34	7.0 (13)
Ox muscle........	16.00	6.50	6.48	5.3 (9), 5.7 (24)
Peanut, total globulins....	18.01	4.28	4.40	
Phaseolin (navy bean).... .	16.07	6.69	6.45	
Wheat bran globulin..	17.76	3.82	3.90	
Zein..	16.00	5.03	5.08	
Barley, pearled	1.86	0.53	0.55	0.45 (14)
Brazil nut meal.....	9.03	2.09	2.01	
Corn germ, defatted	3.93	0.92	0.88	1.0 (24)
Corn, whole, yellow.. . ..	2.22	0.61	0.58	0.89 (14)
Cottonseed flour...	10.36	2.16	2.06	2.20 (5), 2.91 (14), 2.52 (24)
Egg, whole, dried....	8.11	4.43	4.09	3.57 (24), 4.05 (25)
Milk, dry, skim......	6.57	2.51	2.35	2.46 (8), 2.14 (13), 2.96 (14), 2.96 (26), 2.38 (27)
Oatmeal.	2.73	0.76	0.74	0.71 (14), 0.90 (23)
Peanut flour...........	10.15	2.44	2.38	2.16 (5), 2.92 (24)
Peas, black-eyed..............	4.15	1.27	1.23	1.32 (23)
Rice, white.	1.26	0.35	0.33	
Rye, whole..	1.98	0.51	0.47	0.50 (13)
Soy bean flour.	8.85	2.71	2.70	2.60 (5), 2.80 (11), 2.49 (13), 3.39 (14)
Wheat germ, defatted........	6.50	1.46	1.49	1.42 (23), 1.54 (24)
" whole................	3.07	0.69	0.67	0.63 (5), 0.69 (13), 0.83 (14)
Yeast, dried, brewers'........	7.71	2.37	2.42	2.02 (13), 2.90 (24), 2.84 (28)

Recovery of isoleucine added to hydrolysates of arachin, casein, corn, and milk powder was satisfactory for this type of assay with an over-all standard deviation of ±1.8 per cent (Table II).

Table III shows values found for several proteins and foods at different assay levels. Data on the reproducibility of values found for a number

of materials when determined by separate assays on different samples are given in Table IV.

After the results with *L. mesenteroides* were obtained, a second organism *S. faecalis* 9790[1] was used to assay the same materials with the same medium. The standard curve for *S. faecalis* is shown in Fig. 2. The results obtained by the two organisms are given in Table V[2] together with values found by other workers.

SUMMARY

A microbiological method is described for the determination of isoleucine in proteins and foods with *Leuconostoc mesenteroides*. The results obtained were checked by assays with *Streptococcus faecalis* and the same basal medium. The values obtained are in close agreement.

BIBLIOGRAPHY

1. Niven, C. F., Jr., and Sherman, J. M., *J. Bact.*, **47**, 335 (1944).
2. Niven, C. F., Jr., *J. Bact.*, **47**, 343 (1944).
3. Dunn, M. S., Camien, M. N., and Shankman, S., *J. Biol. Chem.*, **161**, 657 (1945).
4. Shankman, S., *J. Biol. Chem.*, **150**, 305 (1943).
5. Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, **151**, 615 (1943).
6. Hegsted, D. M., *J. Biol. Chem.*, **152**, 193 (1944).
7. Schweigert, B. S., Tatman, I. E., and Elvehjem, C. A., *Arch. Biochem.*, **6**, 177 (1945).
8. Hodson, A. Z., and Krueger, G. M., *Arch. Biochem.*, **10**, 55 (1946).
9. Hier, S. W , Graham, C. E., Freides, R., and Klein, D., *J. Biol. Chem.*, **161**, 705 (1945).
10. Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, **166**, 417 (1946).
11. Riesen, W. H., Clandinin, D. R., Elvehjem, C. A., and Cravens, W. W., *J. Biol. Chem.*, **167**, 143 (1947).
12. Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, **156**, 703 (1944).
13. Stokes, J. L., Gunness, M., Dwyer, I. M., and Caswell, M. C., *J. Biol. Chem.*, **160**, 35 (1945).
14. Baumgarten, W., Mather, A. N., and Stone, L., *Cereal Chem.*, **23**, 135 (1946).
15. Smith, E. L., and Greene, R. D., *J. Biol. Chem.*, **167**, 833 (1947).
16. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
17. Smith, E. L., and Greene, R. D., *J. Biol. Chem.*, **172**, 111 (1948).
18. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **176**, 679 (1948).
19. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **166**, 321 (1946).
20. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **169**, 739 (1947).
21. Williamson, M. B., *J. Biol. Chem.*, **156**, 47 (1944).
22. Fontaine, T. D., Olcott, H. S., and Lowy, A., *Ind. and Eng. Chem.*, **34**, 116 (1942).

[2] The sources and preparations of the samples assayed are given in a previous publication on the determination of methionine (29).

23. Block, R. J., and Bolling, D., The amino acid composition of proteins and foods, Springfield (1945).
24. Edwards, L. E., Sealock, R. R., O'Donnell, W. W., Bartlett, G. R., Barclay, M. B., Tully, R., Tybout, R. H., Box, J., and Murlin, J. R., *J. Nutr.*, **32**, 597 (1946).
25. Mitchell, H. H., and Block, R. J., *J. Biol. Chem.*, **163**, 599 (1946).
26. Block, R. J., and Bolling, D., *Arch. Biochem.*, **10**, 359 (1946).
27. Hodson, A. Z., and Krueger, G. M., *Arch. Biochem.*, **12**, 51 (1947).
28. Block, R. J., and Bolling, D., *Arch. Biochem.*, **7**, 313 (1945).
29. Horn, M. J., Jones, D. B., and Blum, A. E., *J. Biol. Chem.*, **166**, 313 (1946).

ISOLATION OF ACETYLHISTAMINE FROM URINE FOLLOW-ING ORAL ADMINISTRATION OF HISTAMINE

By HERBERT TABOR and ERICH MOSETTIG

(*From the Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Maryland*)

An unidentified histamine derivative was demonstrated by Anrep *et al.* (1) in the urine of dogs to which histamine had been administered orally. Rosenthal and Tabor (2) confirmed this observation and noted similarities between this compound and acetylhistamine (4-(β-acetylaminoethyl)imidazole). By means of paper chromatography Urbach (3) demonstrated the identity of the migration rates of a urinary histamine derivative (obtained after oral administration of histamine) and synthetic acetylhistamine. In the present investigation acetylhistamine, isolated from the urine of dogs fed histamine, has been crystallized, characterized, and identified.

EXPERIMENTAL[1, 2]

Synthetic Acetylhistamine and Picrate

4-(β-Acetylaminoethyl)imidazole (Acetylhistamine)—Histamine was acetylated by refluxing it for 1 hour with acetic anhydride according to van der Merwe (4). When pure, the acetylhistamine crystallized readily from absolute ethanol-ether or ethanol-ethyl acetate as colorless prisms. It sublimed readily *in vacuo* (at about 140° and 0.05 mm.). It melted at 147–148°, and was readily soluble in ethanol and in water.

Analysis—$C_7H_{11}N_3O$. Calculated, N 27.4; found, N 27.6

Histamine (base) could be conveniently obtained by passing an approximately 2 per cent solution of histamine dihydrochloride in 95 per cent ethanol through a column of Amberlite IRA-400. The percolate (chloride-free) was evaporated to dryness and the residue purified by sublimation *in vacuo* (at about 100° at 0.05 mm.).

4-(β-Acetylaminoethyl)imidazole Picrate—Acetylhistamine and picric acid (10 per cent in excess of the molecular equivalent) were dissolved in a minimal amount of hot absolute ethanol. Upon cooling, the picrate separated in long prismatic yellow needles. It was recrystallized from

---

[1] All melting points are corrected.

[2] All microanalyses were made in the Microanalytical Service Laboratory of the National Institutes of Health (under the direction of Mr. William C. Alford).

absolute ethanol (if necessary with the addition of ether); m.p. 169–171° (slight sintering at 162°). Upon slow evaporation of an ethanolic solution, the picrate crystallized as yellow prisms, melting at 181–183° (sintering at 175°). The low melting samples changed on standing for 2 to 3 months to the high melting form. By slow cooling of the melt of this modification, the low melting form, m.p. 170–171°, was obtained again.

Analysis—$C_{13}H_{14}N_6O_8$. Calculated, C 40.8, H 3.7; found, C 41.0, H 3.9

For recovery of the base a solution of 50 mg. of the picrate in 10 cc. of absolute ethanol was passed through a column of activated alumina (6 cm. long and 0.8 cm. in diameter), and the column washed with four 10 cc. portions of absolute ethanol. The ethanolic solution was evaporated *in vacuo* and the residue recrystallized and sublimed; m.p. 146–148°.

Administration of Histamine and Recovery of Acetylhistamine from Urine

Experiment 1—660 mg. of histamine dihydrochloride and 600 mg. of $NaHCO_3$ in 150 cc. of milk were administered by stomach tube (in two doses with an interval of 6 hours) to each of three male dogs (weight about 10 kilos) which had been fed a diet of white bread and milk. Morphine sulfate (10 mg. per kilo) had been administered subcutaneously to the dogs half an hour before the histamine dosage (to prevent vomiting). The urine was collected by catheterization during the 36 hour period following histamine administration; voided urines were discarded.

Experiment 2—330 mg. of histamine dihydrochloride and 300 mg. of sodium bicarbonate in 150 gm. of ground horse meat were fed five times weekly for 2 weeks to each of three male dogs. Only catheterized urines were collected.

Experiment 3—The procedure of Experiment 1 was followed, except that voided urines were collected in metabolism cages.

Extraction of Acetylhistamine from Urine—The preliminary purification from the urine was based on the extraction of histamine by *n*-butanol at an alkaline pH, reported by McIntire, Roth, and Shaw (5), and the subsequent reextraction into the aqueous at an acid pH. The content of acetylhistamine[3] at the various stages of purification was estimated colorimetrically (2).

The urine was treated with Na_2SO_4 and $Na_3PO_4 \cdot 12H_2O$ (23 gm. and

---

[3] It has been previously reported that there is only a very small amount of unchanged histamine in the urine of dogs, even after the oral administration of histamine. A quantitative estimation of the total histamine derivative excreted has been reported elsewhere (1, 2, 6). The yields reported in this paper do not represent the total acetylhistamine excreted, since non-catheterized urines were discarded in Experiments 1 and 2 and since there were unavoidable losses during purification.

7 gm. respectively per 100 cc. of urine) and extracted three times with 0.33 volume of *n*-butanol. The combined supernatant butanol layers were extracted four times with 0.066 volume of 0.1 N sulfuric acid. The aqueous acid solution was extracted once with 0.1 volume of butanol (the butanol layer was discarded) and adjusted to pH 8 with sodium hydroxide and phosphate buffer. Na_2SO_4 (23 gm. per 100 cc. of solution) was added, and the mixture was extracted three times with 0.33 volume of butanol. The combined supernatant butanol layers were extracted four times with 0.066 volume of 0.1 N sulfuric acid. This aqueous acid extract is referred to as Solution A.

Isolation, Purification, and Characterization of Acetylhistamine

Experiment 1—Solution A was neutralized with potassium carbonate and evaporated *in vacuo* to dryness. The residue was extracted with absolute ethanol and ethanol-ethyl acetate. Since no well defined fractions were obtained in this manner, the combined extracted material was subjected to fractional sublimation at 0.1 mm. The fraction subliming at 120–180° was recrystallized from ethanol-ether and melted at 135–136° (sintering at 128°). The mixture with an authentic sample of acetylhistamine melted at 134–142°. Yield, approximately 20 mg., corresponding to about 17 per cent of the content of acetylhistamine[3] estimated colorimetrically in the original urine. The product was converted into a picrate melting at 163–165°. The mixture with an authentic acetylhistamine picrate melting at 168–170° melted at 167–169° (softening at 164°). The picrate was decomposed by passing it in an ethanolic solution through alumina. The base melted at 140.5–143° (softening at 135–137°).

Experiment 2—The procedure of isolation was essentially the same as in Experiment 1. In the final sublimation (at 0.05 mm.) two fractions, A and B, were collected, subliming at 80–120° and at 120–170° respectively. Fraction A weighed approximately 15 mg. (corresponding to 15 per cent[3] of the content of histamine derivative in the original urine). The picrate prepared from it melted at 165.5–167.5° (softening at 163°). The mixture with an authentic low melting picrate melted at 165–168° (sintering at 163°). By recrystallization from ethanol the higher melting modification was obtained, melting at 178–180.5° (softening at 175–178°). The mixture of this compound with an authentic high melting sample melted at 179–182°. The resolidified picrate melted at 168–169° (softening at 167°). Fraction B weighed approximately 10 mg. It melted at 140–144.5° (softening at 138.5°); the mixture with an authentic acetylhistamine melted at 144.5–147° (softening at 143.5°). The picrate melted at 167–168° (softening at 163°); the mixture with an authentic low melting picrate melted at 168–169° (softening at 167°). By recrystallization both

modifications were obtained; one melted at 168–169°, the other at 180–181°. The latter after solidification melted at 169–169.5°.

Analysis—$C_{13}H_{14}N_6O_8$.   Calculated, C 40.8, H 3.7; found, C 41.1, H 3.9

Experiment 3—The urine was treated as in Experiments 1 and 2 with minor modifications.   Colorless prismatic needles, melting at 133–139°, were obtained.   The mixture with an authentic sample of acetylhistamine melted at 136–145°.   Yield, 10 mg. (corresponding to about 5 per cent[3] of the acetylhistamine estimated colorimetrically in the original urine).

Analysis—$C_7H_{11}N_3O$.   Calculated, C 54.9, H 7.2; found, C 55.1, H 7.4

SUMMARY

Crystalline 4-(β-acetylaminoethyl)imidazole (acetylhistamine) has been isolated from dog urine after oral administration of histamine.

The product was characterized and identified as the base and the picrate. The latter exists in two crystalline forms.

BIBLIOGRAPHY

1. Anrep, G. V., Ayadi, M. S., Barsoum, G. S., Smith, J. R., and Talaat, M. M., *J. Physiol.*, **103**, 155 (1944).
2. Rosenthal, S. M., and Tabor, H., *J. Pharmacol. and Exp. Therap.*, **92**, 425 (1948).
3. Urbach, K., *Proc. Soc. Exp. Biol. and Med.*, **70**, 146 (1949).
4. van der Merwe, P., *Z. physiol. Chem.*, **177**, 308 (1928).
5. McIntire, F. C., Roth, L. W., and Shaw, J. L., *J. Biol. Chem.*, **170**, 537 (1947).
6. Millican, R. C., Rosenthal, S. M., and Tabor, H., *J. Pharmacol. and Exp. Therap.*, in press.

THE RATE OF UTILIZATION OF AMMONIA FOR PROTEIN SYNTHESIS*

By DAVID B. SPRINSON AND D. RITTENBERG

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

The ability of rats to utilize dietary ammonia for the synthesis of amino acids has previously been demonstrated (1, 2). Following the administration of labeled ammonia to these animals N^{15} was found in most of the amino acids of the tissue proteins.

In order to study the effect of dietary conditions on the utilization of ammonia we have administered a small single dose of ammonium citrate of high N^{15} concentration to both rats and human subjects maintained at various levels of nitrogen output. Since the ammonia nitrogen which is not employed for protein synthesis is almost completely excreted in the urine, the rate of excretion will reflect the rate of utilization of ammonia nitrogen for protein synthesis. The results obtained are shown in Fig. 1. In the case of rats on a high protein diet (Experiments S-61, S-62, and S-63 of Table I) the administered ammonia is almost quantitatively excreted, while on a low protein diet (Experiments S-51 and S-53) ammonia nitrogen is incorporated into proteins almost as efficiently as glycine nitrogen on a 15 per cent casein diet (3). Following the ingestion of labeled ammonium citrate by a human subject on a normal diet, 61 per cent of the ammonia nitrogen is excreted in 24 hours and 74 per cent in 48 hours (Table II).

In comparison with amino acids, ammonia is poorly utilized for protein synthesis; it is extensively utilized only when there is a deficiency of dietary amino acids. In part this is due to the fact that it is rapidly converted in the liver to urea, which is unavailable for synthetic reactions (4).

It appears that only a small portion of the absorbed ammonia reaches the kidney and is excreted as such. If the reverse were true, then the isotope concentration of the urinary ammonia during the first 3 hours of the experiment would be higher than that of the urea. Such is not the case,
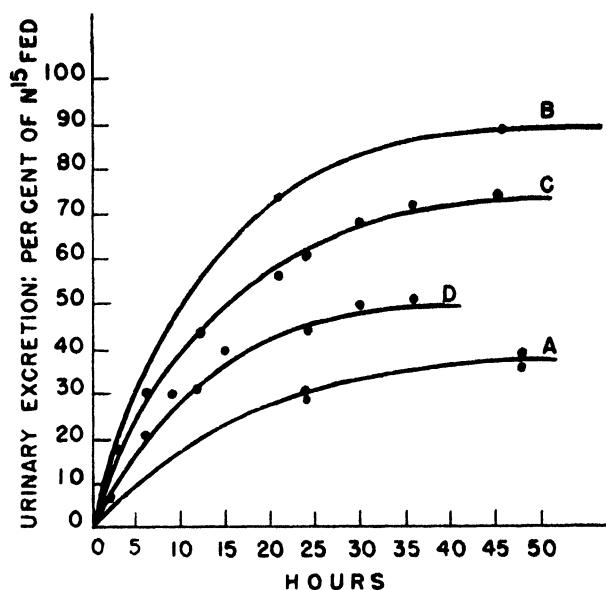
Fig. 1. Effect of protein intake on excretion of ammonia nitrogen. Curve A, rats on a low protein diet; Curve B, rats on a high protein diet; Curve C, a human subject on a normal diet; Curve D, a human subject on a low protein diet (Addison's disease).

TABLE I

Excretion of Ammonia Nitrogen by Rats on Low and High Protein Diets

Diet	Experiment No.	Weight of rat	Time	Total N excretion per 24 hrs.	N¹⁵ concentration	N¹⁵ excreted	ΣN¹⁵† excreted	Excretion of fraction of N¹⁵ fed
		gm.	*hrs.*	*gm.*	*atom per cent excess*	*m.eq*	*m.eq.*	*per cent*
ow protein	S-51	230	0–24	0.067	0.63	0.030	0.030	29
			24–48	0.100	0.16	0.011	0.041	39
" "	S-53	280	0–24	0.071	0.64	0.033	0.033	31
			24–48	0.062	0.12	0.005	0.038	36
igh "	S-61	330	0–24	1.47	0.078	0.082	0.082	78
			24–48	1.51	0.01	0.010	0.092	88
" "	S-62	350	0–24	1.66	0.083	0.099	0.099	94
" "	S-63	320	0–24	1.42	0.10	0.101	0.101	96

* 0.105 m.eq. of N¹⁵ as ammonium citrate (containing 32 atom per cent excess N¹⁵) was administered at the start of the experiment.

† In this column are given, for any individual experiment, the sums of the N¹⁵ excreted since zero time.

for the N¹⁵ concentration of the urinary ammonia is always less than that of the urea (see Tables II and III).

TABLE II

Excretion of Ammonia Nitrogen by Normal Human Subject*

ime	Total N	N¹⁵ concentration in urinary constituents			Total N¹⁵ excreted	ΣN¹⁵† excreted	Excretic of fracti of N¹⁵ fe
		Total N	Urea	Ammonia			
rs.	*gm.*	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*	*m.eq.*	*m.eq.*	*per cent.*
⊢ 3	1.72	0.202	0.226	0.196	0.248	0.248	17.6
⊢ 6	1.55	0.156	0.186	0.042	0.173	0.421	29.9
⊢12	2.97	0.093	0.125	0.018	0.197	0.618	43.8
⊢21	4.23	0.055	0.069	0.016	0.166	0.784	55.6
-24	1.97	0.051	0.058	0.015	0.072	0.856	60.7
-30	3.26	0.043	0.040	0.014	0.100	0.956	67.8
⊢36	3.58	0.022	0.030	0.008	0.056	1.01	71.6
-45	5.15	0.009	0.014	0.011	0.033	1.04	73.8

* 1.41 m.eq. of N¹⁵ as ammonium citrate (containing 18.3 atom per cent excess N¹⁵) were ingested at the start of the experiment. The weight of the subject was 64.4 kilos. A normal diet was consumed.

† In this column are given the sums of the N¹⁵ excreted since zero time.

TABLE III

Excretion of Ammonia Nitrogen by Human Subject on Low Protein Diet (Addison's Disease)*

Time	Total N	N¹⁵ concentration in urinary constituents			Total N¹⁵ excreted	ΣN¹⁵† excreted	Excretion of fraction of N¹⁵ fed
		Total N	Urea	Ammonia			
hrs.	*gm.*	*atom per cent excess*	*atom per cent excess*	*atom per cent excess*	*m.eq.*	*m.eq.*	*per cent*
−1 to 2‡	0.429	0.266	0.369	0.302	0.082	0.082	7.3
2− 6	0.496	0.448	0.723	0.236	0.158	0.240	21.2
6− 9	0.402	0.338	0.576	0.176	0.097	0.337	29.8
9−12	0.094	0.261	0.464	0.080	0.018	0.355	31.4
12−15	0.615	0.223	0.368	0.057	0.098	0.453	40.1
15−24	0.528	0.127	0.218	0.045	0.048	0.501	44.3
24−30	0.892	0.102	0.173	0.040	0.065	0.566	50.1
30−36	0.810	0.021	0.096	0.032	0.012	0.578	51.1

* 1.13 m.eq. of N¹⁵ as ammonium citrate (containing 18.3 atom per cent excess N¹⁵) were ingested at the start of the experiment. The weight of the subject was 54 kilos.

† In this column are given the sums of the N¹⁵ excreted since zero time.

‡ Urine collection was begun 1 hour before administration of the labeled glycine.

In contrast to the results with ingested ammonia, the feeding of an amino acid, labeled with N¹⁵, results initially in a higher isotope concentration in the urinary ammonia than in the urea. In Table IV are shown typical

data from experiments in which isotopic glycine was administered to human subjects. Experimental details are given in the following paper (3) (Experiments DS-2, MZ-1, GL-1). Here, during the first 3 hours, the isotope concentration in the ammonia is greater than in the urea, suggesting that some of the isotopic glycine passed through the liver and was available in the kidney for ammonia formation. After 3 hours the N^{15} concentration in the ammonia is less than that of the urea; glycine containing a high N^{15} concentration is no longer available to the kidney.

In previous experiments (5) with rats in which labeled compounds were incorporated into the diet the ratio of the N^{15} concentration of the ammonia to that of the urea was less than 1 when ammonia was fed and greater

TABLE IV

N^{15} Concentrations in Urinary Constituents Following Administration of Labeled Glycine to Normal Human Subjects*

Experiment No.	Time	Total N	Urea	Ammonia	$\dfrac{N^{15} \text{ in NH}_3}{N^{15} \text{ in urea}}$
	hrs.	atom per cent excess	atom per cent excess	atom per cent excess	
MZ-1	0– 3	0.142	0.139	0.247	1.77
	3– 7	0.106	0.108	0.049	0.45
	7–15	0.072	0.079	0.035	0.44
	15–24	0.052	0.057	0.024	0.42
GL-1	0– 3	0.155	0.123	0.353	2.87
	3– 7	0.106	0.118	0.054	0.46
DS-1	0– 3	0.135	0.100	0.554	5.54
	3– 6	0.112	0.124	0.122	0.98
	6– 9	0.099	0.106	0.079	0.74
	9–12	0.070	0.080	0.040	0.50
	12–21	0.050	0.061	0.033	0.54

* See the following paper (3) for the experimental details.

than 1 when an amino acid was fed. When the labeled compound was continuously supplied in the diet, a condition analogous to that of the initial period (0 to 3 hours) of the present experiments was simulated, so that the excretion pattern of the N^{15} is essentially the same for both types of feeding.

Through the kindness of Dr. R. F. Loeb we had the opportunity to study ammonia utilization in a patient suffering from Addison's disease. This patient was eating very little, her total nitrogen output per day being about 2.5 gm. In this case of prolonged nitrogen starvation the utilization of ammonia nitrogen (see Table III) was almost as high as the utilization of glycine nitrogen by human subjects on a normal diet (3).

It is to be noted that the data presented here relate to experiments in which small amounts of ammonia were administered, and the conclusions

derived need not necessarily hold when large amounts of ammonia are administered.

Ammonium citrate was prepared by distilling ammonia containing 32 atom per cent excess N^{15} into an aqueous solution of 1 mole of citric acid; it was administered by mouth in one dose. The rats were given diets of the following percentage composition: low protein diet, starch 83, brewers' yeast 5, salt mixture (6) 4, cottonseed oil (Wesson oil) 6, cod liver oil 2; high protein diet, casein 81 (Eimer and Amend), Celluration 2, and the remaining ingredients as above. Urine was collected and analyzed for urea, ammonia, and total nitrogen by the usual procedures. The N^{15} concentration was determined by a modification of our previous methods (7, 8).

After the sample had been subjected to Kjeldahl digestion for 18 hours, the ammonia was liberated and distilled into 5 cc. of 0.07 N H_2SO_4 (8). The distillate was then boiled down to about 2 cc.

Oxidation of ammonia to nitrogen was effected by hypobromite. The hypobromite solution was prepared by the *slow* addition, with vigorous stirring, of 50 cc. of bromine to 150 cc. of 40 per cent (by weight) reagent quality NaOH held at 0°. Since hypobromite decomposes less readily in strongly alkaline solutions, another 150 cc. of 40 per cent NaOH were added when the addition of bromine was completed. 1 cc. of this solution should oxidize from 10 to 12 mg. of NH_3. When stored in a refrigerator, it retains its activity for months.

Freshly prepared hypobromite tends to liberate oxygen, but this effect generally disappears after the reagent has been allowed to stand for a few days. After this time the solution can be separated by decantation from the precipitated sodium salts. Occasionally a batch of hypobromite is obtained which continues to exhibit this decomposition. The cause is not known, but it can be demonstrated that the rate of evolution of oxygen is accelerated by cupric ions.

We have studied the effect of several substances on the rate of decomposition of a sample of hypobromite which continuously evolved oxygen. The data, obtained manometrically, are given in Table V. It is clear that contamination with copper of either the reagent or the ammonia sample must be avoided. For this reason $HgSO_4$ rather than $CuSO_4$ should be employed as a catalyst in the Kjeldahl procedure.

Before use the hypobromite is diluted with an equal volume of water. Approximately 3 cc. of the diluted hypobromite solution are placed in one arm of the Y-tube[1] shown in Fig. 2, and the ammonium sulfate sample is pipetted into the other arm. The joint is greased, the cap put into posi-

[1] This vessel can be obtained from Eck and Krebs, 131 West 24th Street, New York.

tion, and the vessel is connected to a vacuum system (Fig. 3) by the 10/30 ground joint. Several tubes may be connected at the same time. Following evacuation by a mercury diffusion pump to less than 0.01 mm. as in-

TABLE V

Effect of Various Ions on Rate of Decomposition of Hypobromite at 25°

A manometer vessel containing 3 cc. of the hypobromite reagent and the indicated addition was shaken for 1 hour.

Addition	O_2 evolved per hr.	Addition	O_2 evolved per hr.
	c mm.		*c.mm.*
None	41	5.0 mg. $HgCl_2$	26
1.0 mg. $CuSO_4$	4050	0.1 " $Na_3AsO_4 \cdot 7H_2O$	61
0.1 " "	140	10 " $KBrO_3$	41
0.1 " " + 10 mg. NaF	152	0.1 " $FeCl_3 \cdot 6H_2O$	30
0.1 " $HgCl_2$	29		
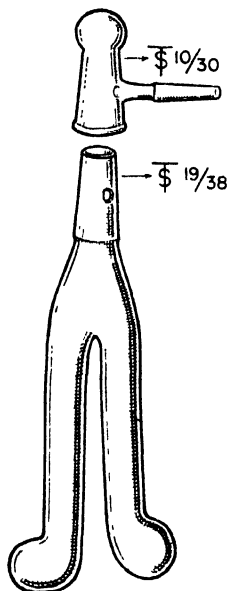
FIG. 2. Gas sample tube for oxidation of ammonium salts with hypobromite

dicated by a Pirani gage, the tubes are disconnected from the pumping system by rotating the Y-tube and detached.

Prior to mass spectrometric analysis the ammonia sample is oxidized to nitrogen by mixing the hypobromite and the sample. After nitrogen

evolution has ceased, the Y-tube is placed in a dry ice-alcohol mixture to reduce the vapor pressure of the solution to a low value. The vessel is then attached to the gas line of the mass spectrometer by means of the 10/30 joint and the nitrogen sample admitted by suitably turning the Y-tube in relation to the cap.

These vessels will ordinarily show no leakage of air after several hours, especially if tubes and caps are not interchanged but kept permanently paired. However, if they are stored overnight in the refrigerator before mixing, they should be reevacuated. Routine preparation of samples yields nitrogen contaminated by less than 0.25 per cent of air as indicated by the
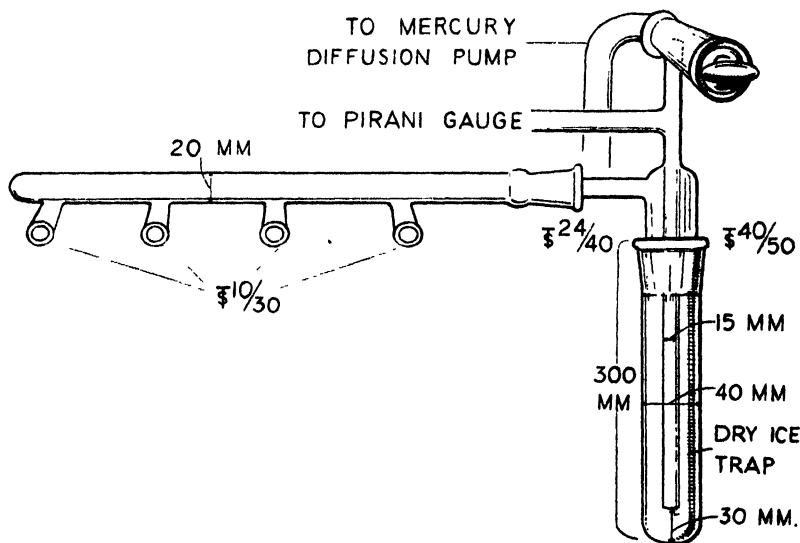
Fig. 3. Vacuum system for preparation of gas samples for mass spectrometric analysis.

intensity of the argon peak. Most samples contain less than 0.1 per cent of air. This procedure yields results which are superior to those obtained by previous methods, and with a considerable reduction in time. With a sufficient number of vessels, sixteen samples can be prepared per hour.

The same tubes can be employed for the preparation of CO_2 for either C^{13} or O^{18} determinations.

SUMMARY

The rate of utilization of dietary ammonia nitrogen for protein synthesis was measured by determining the rate of excretion of the labeled nitrogen. On a high protein diet ammonia nitrogen is nearly quantitatively excreted;

almost none is utilized. On very low protein diets an appreciable fraction of the ammonia nitrogen is employed for protein synthesis.

The relationship between the isotope concentrations of the urinary ammonia and urea following administration of labeled ammonia or amino acids is discussed.

A modified procedure for the preparation of gas samples for mass spectrometric analysis is described.

We are indebted to Miss L. Ponticorvo and Mr. I. Sucher for technical assistance and to Dr. M. Rothenberg for assistance in carrying out the manometric experiments.

BIBLIOGRAPHY

1. Foster, G. L., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **127**, 319 (1939).
2. Rittenberg, D., Schoenheimer, R., and Keston, A. S., *J. Biol. Chem.*, **128**, 603 (1939).
3. Sprinson, D. B., and Rittenberg, D., *J. Biol. Chem.*, **180**, 715 (1949).
4. Bloch, K., *J. Biol. Chem.*, **165**, 469 (1946).
5. Schoenheimer, R., The dynamic state of body constituents, Cambridge, 51 (1942).
6. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).
7. Rittenberg, D., Keston, A. S., Rosebury, F., and Schoenheimer, R., *J. Biol. Chem.*, **127**, 291 (1939).
8. Rittenberg, D., in Wilson, D. W., Nier, A. D. C., and Reimann, S. P., Preparation and measurement of isotopic tracers, Ann Arbor (1946).

THE RATE OF INTERACTION OF THE AMINO ACIDS OF THE DIET WITH THE TISSUE PROTEINS*

By DAVID B. SPRINSON AND D. RITTENBERG

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

In an adult animal in nitrogen balance the loss of nitrogen is equal to the dietary intake. The initial experiments with N^{15}-labeled amino acids showed that the nitrogen atoms excreted were not solely those contained in the diet. These results were not consistent with the view that the tissue proteins are chemically inert. On the contrary, it appeared that the proteins and amino acids were continually being degraded and resynthesized. The steady state of the organism appeared to be a reflection of the equality of the rates at which these chemical reactions occur. The view of the living cell as a dynamic system, elaborated in 1939 by Schoenheimer, Ratner, and Rittenberg (1), involved the concept of a metabolic pool.

This theory has been generally accepted. That tissue constituents interact with the diet at a rapid rate has been demonstrated for most components of the diet (2). However, neither the magnitude of the interactions, e.g. the rate of protein synthesis, nor the size of the metabolic pool or pools has as yet been estimated in the intact animal. Indeed the metabolic pool has never been exactly defined except by implication in the paper cited (1). We shall here define the metabolic pool of the animal (or organ or cell) as that mixture of compounds, derived either from the diet or from the breakdown of the tissues, which the animal (or organ or cell) employs for the synthesis of tissue constituents. The nitrogenous compounds of the metabolic pool constitute the nitrogen pool.

It is probable that all of the constituents of the nitrogen pool are present in the non-protein fraction. The reverse is not true. Urea and creatinine for example are not part of the metabolic pool, since they are solely excretory products (3, 4). For this reason neither the magnitudes nor the isotope concentrations of the nitrogen pool and the non-protein fraction are necessarily the same.

When an amino acid is administered to an animal, it mixes with the metabolic pool. Part of the nitrogen is used for protein synthesis and another part is converted to the excretory products and excreted. The more rapid and extensive the utilization of an isotopic nitrogen compound for protein synthesis, the less labeled nitrogen will be found in the urine. The rate of excretion of N^{15} will thus be a measure of the utilization of nitrogen of the compound fed for protein synthesis and of the magnitude
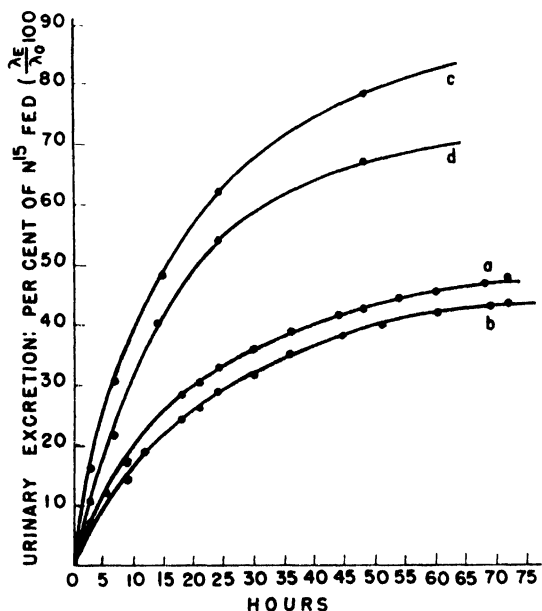
Fig. 1. Effect of protein intake on excretion of glycine nitrogen. Curves a and b, human subjects on a normal diet (about 0.20 gm. of nitrogen per kilo of body weight excreted in 24 hours). Curves c and d, human subjects on a high protein diet (about 0.54 gm. of nitrogen per kilo of body weight excreted in 24 hours).

of the metabolic pool. We have attempted to evaluate the magnitudes of these quantities.

Normal human subjects in nitrogen equilibrium on a diet of their own choosing were given by mouth in a single dose 10 mg. of N^{15}-labeled glycine per kilo of body weight. The rate of urinary excretion of N^{15} for two typical experiments is shown in Curves a and b in Fig. 1. About 30 per cent of the labeled nitrogen was excreted during the first 24 hours, and about 40 per cent in the first 48 hours. The excretion rate at this time had fallen to a very low level. It seems clear that on a normal diet approximately 70 per cent of the dietary glycine nitrogen is used for the formation

of tissue proteins. These results are in agreement with those previously obtained in rats on a 15 per cent casein diet supplemented with labeled glycine (5). The proportion of retained nitrogen is, however, not an unalterable constant. The degree of interaction of dietary amino acid nitrogen with body protein can be greatly influenced by the level of nitrogen intake. On a high protein diet glycine is poorly utilized (Curves c and d, Fig. 1), since nearly twice as much of the amino acid nitrogen is excreted as on a normal diet.

We have found empirically that the excretion of the labeled glycine nitrogen in both the rat and the human can be represented by Equation 1,

$$\lambda_E = \lambda_0 A (1 - e^{-Bt}) \tag{1}$$

where λ_E = the amount of N^{15} excreted up to time t (in days), λ_0 = the amount of N^{15} fed, and A and B are constants. Equation 1 can be transformed as follows:

$$\frac{\lambda_E}{\lambda_0 A} = 1 - e^{-Bt} \tag{2}$$

$$e^{-Bt} = 1 - \frac{\lambda_E}{\lambda_0 A} \tag{3}$$

$$-Bt = \ln\left(1 - \frac{\lambda_E}{\lambda_0 A}\right) = 2.3 \log\left(1 - \frac{\lambda_E}{\lambda_0 A}\right) \tag{4}$$

The values of A and B can be calculated from this equation. The closeness with which the data of any one experiment fit Equation 4 is shown in Fig. 2 where the results of two typical human experiments, DR-1 and DR-2, are plotted in a semilogarithmic coordinate system. The data for these experiments are given in Tables I and II. The values of A and B for all human experiments are given in Table III.[1]

The relationship of the constants A and B to the size of the metabolic pool and the rate of protein synthesis can be developed formally on the basis of certain assumptions. Assume that there is but one homogeneous metabolic pool containing P gm. of nitrogen. Let dietary nitrogen enter the pool at a constant rate of D gm. per day and nitrogen from breakdown of proteins enter at a constant rate of R gm. per day. Nitrogen leaves the metabolic pool by two routes; S gm. per day are used for the synthesis of tissue proteins and E gm. per day for the excretory products. If, as we assume, the animal is in a stationary state, $S = R$ and $D = E$.

Let λ_0 m.eq. of N^{15}, as amino acid, be introduced at zero time and λ be

---

[1] We assume that all of the N^{15} excreted is in the urine. Actually a few per cent of the N^{15} is excreted in the feces (1). This will tend to make our values for total nitrogen excretion and λ_E low, and so the values of A will also be low.
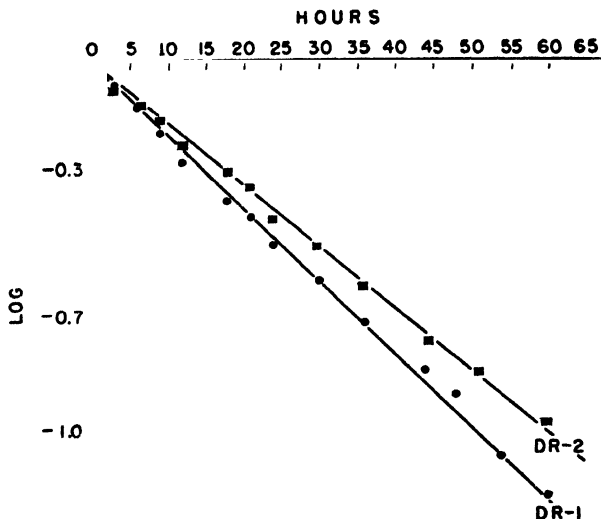
FIG. 2. Utilization of glycine nitrogen in human subjects as represented by Equation 4; see Tables I and II.

TABLE I

Rate of Utilization of Glycine Nitrogen by Human Subject (Experiment DR-1)

Time	Total N excretion	N^{15} concentration	λ_E*	$\Sigma\lambda_E$	$\dfrac{\lambda_E}{\lambda_0*}$	$1 - \dfrac{\lambda_E}{\lambda_0 A}$ †
hrs.	*gm.*	*atom per cent excess*	*m.eq.*	*m.eq.*		
0- 3	1.70	0.176	0.214	0.214	0.073	0.85
3- 6	1.55	0.133	0.147	0.361	0.123	0.75
6- 9	1.91	0.113	0.154	0.515	0.175	0.64
9-12	2.51	0.088	0.158	0.673	0.229	0.53
12-18	3.18	0.072	0.164	0.837	0.285	0.42
18-21	1.29	0.062	0.057	0.894	0.304	0.38
21-24	1.85	0.057	0.075	0.969	0.330	0.32
24-30	2.58	0.048	0.088	1.06	0.360	0.26
30-36	3.34	0.035	0.083	1.14	0.389	0.20
36-44	3.54	0.029	0.073	1.21	0.415	0.15
44-48	1.64	0.025	0.029	1.24	0.425	0.13
48-54	3.31	0.022	0.052	1.29	0.443	0.09
54-60	2.46	0.018	0.032	1.33	0.453	0.07

* $\lambda_E = N^{15}$ excreted; $\lambda_0 = N^{15}$ fed at zero time = 2.92 m.eq.
† $A = 0.488$ (see the text).

the amount of N^{15} remaining in the pool at any time t. Let λ_0 be very small relative to P. Then

$$-d\lambda = \frac{\lambda}{P} E\,dt + \frac{\lambda}{P} S\,dt - Q\,dt \qquad (5)$$

The first quantity, $(\lambda/P)Edt$, on the right side of Equation 5 represents the amount of N^{15} excreted in the time dt, for Edt is the amount of nitrogen excreted in time dt and λ/P is the atom fraction of N^{15} in the pool. The second quantity, $(\lambda/P)Sdt$, is the amount of N^{15} removed from the metabolic pool for protein synthesis. Q represents the rate at which N^{15} is delivered to the metabolic pool as a result of the breakdown of protein which during the course of the experiment has incorporated N^{15}. We shall disregard Q $(Q = 0)$, for it is clear that when, as in this case, λ_0 is very much smaller than the total nitrogen content of the animal Q will be negligible with respect to either E or S.

TABLE II

Rate of Utilization of Glycine Nitrogen by Human Subject (Experiment DR-2)

Time	Total N excretion	N^{15} concentration	λ_E*	$\Sigma\lambda_E$	$\dfrac{\lambda_E}{\lambda_0^*}$	$1 - \dfrac{\lambda_E}{\lambda_0 A}$ †
hrs.	*gm.*	*atom per cent excess*	*m.eq.*	*m.eq.*		
0–3	1.76	0.164	0.216	0.216	0.074	0.84
3–6.5	1.12	0.132	0.106	0.322	0.110	0.76
6.5–9	1.23	0.117	0.103	0.425	0.145	0.69
9–12	2.03	0.092	0.133	0.558	0.191	0.59
12–18	2.72	0.078	0.151	0.709	0.243	0.50
18–21	1.20	0.060	0.051	0.760	0.261	0.46
21–24	1.78	0.065	0.083	0.843	0.289	0.38
24–30	2.18	0.054	0.084	0.927	0.317	0.32
30–36	3.58	0.037	0.095	1.02	0.350	0.25
36–44.5	5.09	0.024	0.087	1.11	0.380	0.18
44.5–51	3.89	0.019	0.053	1.16	0.398	0.15
51–60	5.38	0.014	0.054	1.22	0.418	0.11

* λ_E = N^{15} excreted; λ = N^{15} fed at zero time = 2.92 m.eq.

† A = 0.466 (see the text).

It is to be noted that either E or S can have many components; *i.e.*, nitrogen from the metabolic pool may be used for many synthetic processes having rates of $s_1, s_2, \ldots s_n$ or excretory processes having rates $e_1, e_2, \ldots e_n$. Under these conditions $S = \Sigma s_n$ and $E = \Sigma e_n$.

Equation 5 can now be written as

$$\frac{d\lambda}{\lambda} \cdot \frac{E + S}{} dt \qquad (6)$$

and integrated to yield

$$\ln \lambda = -\left(\frac{E + S}{P}\right) t + \text{constant} \qquad (7)$$

The constant of integration is equal to $\ln \lambda_0$, for at $t = 0$, $\lambda = \lambda_0$. On making this substitution, Equation 8 is obtained.

$$\lambda_0 \, e^{-[(B+S)/P]t} \tag{8}$$

TABLE III

Rate of Protein Synthesis and Size of Nitrogen Pool in Human Subjects

| Experiment No. | Weight of subject | E^* | $100 \dfrac{\lambda_E}{\lambda_0}$ † | | A | B | S^* | P^* | E_k^* | S_k^* | P_k^* |
			0–24 hrs.	0–48 hrs.							
	kg.	*gm.*	*per cent*	*per cent*			*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
DS-1	75.0	17.2	34.7	42.0	0.450	1.21	21.0	31.6	0.229	0.280	0.421
DS-2	75.0	16.3	27.0	35.0	0.382	1.27	26.4	33.6	0.217	0.352	0.448
DR-1	64.4	14.0	33.0	42.5	0.488	1.08	14.7	26.6	0.217	0.228	0.412
DR-2	64.4	11.9	28.9	39.0	0.466	0.91	13.7	28.1	0.185	0.213	0.429
MZ-1	57.6	31.1	62.0	82.9	0.926	1.18	2.5	28.5	0.540‡	0.043‡	0.494
MZ-2	57.6	9.4	18.1	29.1	0.500	0.45	9.4	41.9	0.163	0.163	0.728
MZ-3	57.6	12.2	25.6	43.3	0.650	0.52	6.6	36.3	0.212	0.115	0.630
MZ-4	57.6	12.4	31.0	43.8	0.556	0.79	9.9	28.2	0.215	0.172	0.490
GL-1	77.2	41.1	53.8	67.2	0.724	1.33	15.7	42.7	0.532‡	0.203‡	0.553
GL-2	77.2	16.1	24.7	34.3	0.420	0.85	22.3	45.2	0.209	0.289	0.585
JE-1	63.5	10.4	22.4	32.9	0.457	0.72	12.4	31.7	0.164	0.195	0.500
JE-2	63.5	11.4	26.9	38.4	0.500	0.78	11.4	29.2	0.180	0.179	0.460
Average					. . . . .	. . .			0.199 ±0.020	0.218 ±0.054	0.514 ±0.074

* This is the total nitrogen excretion during the first 24 hours of the experiment and was used to calculate S and P by means of Equations 17 and 18. The values of S (gm. of N synthesized into protein per 24 hours) and P (gm. of N in the metabolic pool) were not materially changed when the average daily excretion was taken as E. E_k, S_k, and P_k are the values of E, S, and P per kilo of body weight.

† The complete data for λ_E/λ_0 (the fraction of N[15] fed which is excreted in the urine) are shown for two typical experiments in Tables I and II and Fig. 2.

‡ Because of the high dietary nitrogen these values of E_k and S_k were omitted from the average. The size of the pool did not vary appreciably on these high protein diets.

Equation 8 can now be employed to calculate the N[15] concentration of the pool.

The isotope concentration of the metabolic pool, N_P, at any instant is

$$N_P = 100 \frac{\lambda}{P} = 100 \frac{\lambda_0}{P} \left(e^{-[(B+S)/P]t} \right) \tag{9}$$

The average amount of N^{15} present in the pool, $\bar{\lambda}$, from the time $t = t_1$ to time $t = t_2$ is

$$\bar{\lambda} = \frac{\int_{t_1}^{t_2} \lambda \, dt}{\int_{t_1}^{t_2} dt} = \frac{\int_{t_1}^{t_2} \lambda_0 (e^{-[(E+S)/P]t}) \, dt}{\int_{t_1}^{t_2} dt} \tag{10}$$

$$\bar{\lambda} = \frac{\left[-\dfrac{P}{E+S} \lambda_0 (e^{-[(E+S)/P]t}) \right]_{t_1}^{t_2}}{[t]_{t_1}^{t_2}} . \tag{11}$$

$$\bar{\lambda} = \frac{\dfrac{P\lambda_0}{E+S} (e^{-[(E+S)/P]t_1} - e^{-[(E+S)/P]t_2})}{t_2 - t_1} \tag{12}$$

The average N^{15} concentration of the pool, $\bar{N}_P$, for this interval is, therefore,

$$\bar{N}_P = 100 \frac{\bar{\lambda}}{P} = \frac{100\lambda_0}{(E+S)(t_2 - t_1)} (e^{-[(E+S)/P]t_1} - e^{-[(E+S)/P]t_2}) \tag{13}$$

The values for S and P can be estimated as follows: Let λ_E be the amount of N^{15} excreted in milliequivalents. Then

$$d\lambda_E = \frac{\lambda}{P} E \, dt \tag{14}$$

Substituting for λ from Equation 8,

$$d\lambda_E = \lambda_0 \frac{E}{P} (e^{-[(E+S)/P]t}) \, dt \tag{15}$$

Integrating and evaluating the constant of integration from the boundary condition $\lambda_E = 0$ at $t = 0$ yields

$$\lambda_E = \lambda_0 \frac{E}{E+S} (1 - e^{-[(E+S)/P]t}) \tag{16}$$

Comparison of Equation 1, which represents our data, with Equation 16 reveals that

$$A = \frac{E}{E+S} \tag{17}$$

and

$$B = \frac{E+S}{P} \tag{18}$$

From the values of A, B, and E for any particular experiment we can evaluate S and P. These values of S and P are given in Table III. We have also calculated E_k, S_k, and P_k which are the values of E, S, and P per kilo body weight.

If, as seems quite likely, urea is directly derived from the nitrogen of the metabolic pool, the isotope concentration of the urea formed at any instant, and its average isotope concentration over a period of time from t_1 to t_2, should be given by Equations 9 and 13 respectively. Using Equation 13 and the values of E, S, and P, we have calculated the isotope concentrations to be expected in the urea and compared these values with those found in the urinary urea (see Table IV). They are in as good agreement as

TABLE IV

Average Isotope Concentration of Nitrogen Pool

Experiment No.	Time after glycine feeding		Average N^{15} concentration calculated by Equation 13	N^{15} concentration of urinary urea
	t_1	t_2		
	hrs.	*hrs.*	*atom per cent excess*	*atom per cent excess*
DS-2	0	3	0.129	0.100
	3	6	0.110	0.124
	6	9	0.094	0.106
	9	12	0.080	0.080
	12	21	0.059	0.061
DR-2	0	3	0.152	0.127
MZ-1	0	3	0.145	0.139
	3	7	0.120	0.108
	7	15	0.091	0.079
	15	24	0.060	0.057
GL-1	0	3	0.128	0.123
	3	7	0.107	0.118
	7	14	0.080	0.104

could be expected in view of the fact that urea, once formed, is not immediately excreted.

The values of S_k range from 0.043 to 0.352 gm. per day. Excluding Experiments MZ-1 and GL-1, in which E was unusually high, we find in those subjects who took their normal diet an average value for S_k of 0.218 ± 0.054. Since the average protein nitrogen content of human tissue is 25 gm. per kilo (6), this value of S_k indicates a protein turnover of only 0.87 per cent (0.218/25.0 × 100) per day. If the process is truly random, the half life time, $t_{\frac{1}{2}}$, can be calculated from Equation 19

$$kt_{\frac{1}{2}} = \ln 2 = 0.693 \tag{19}$$

where k is the fraction of protein degraded and resynthesized per day. For $k = 0.0087$ the value of $t_{\frac{1}{2}}$ is equal to 80 days.

Some tissues in the human organism have a much shorter half life time than 80 days. The serum proteins have a half life time of about 10 days.[3] There is another organ, the liver, which almost surely has approximately the same turnover time as the blood proteins (7). In a 70 kilo man containing 1750 gm. of protein nitrogen the plasma and the liver proteins account for 90 gm. of nitrogen (6). If the value of $t_{\frac{1}{2}}$ of both tissues is taken to be 10 days, then the fraction of protein synthesized per day, calculated from Equation 19, is 0.0693, and the daily rate of protein synthesis in these two organs is equivalent to 6.2 gm. of nitrogen (90 $\times$ 0.069). For a 70 kilo man the rate of protein synthesis is equivalent to 70 $\times$ 0.218 = 15.3 gm. of nitrogen. These calculations indicate that almost half (41 per cent) of all protein synthesis in man is associated with liver tissue and plasma proteins. If, as is generally believed, the plasma proteins are synthesized in the liver, the latter organ accounts for nearly half of the protein synthesis in the entire organism.

The proteins of the internal organs (spleen, heart, kidneys, intestine, testes) in the rat have values of $t_{\frac{1}{2}}$ close to that for the liver (8). It is likely that the same is true for the human. The nitrogen content of spleen, heart, kidneys, small intestine, pancreas, and endocrine glands in a 70 kilo man is approximately 60 gm. (6). Even if we assume an average value of $t_{\frac{1}{2}} = 20$ days for these tissues, they would utilize 2.1 gm. (0.69/20 $\times$ 60) of nitrogen per day for protein synthesis. On this assumption, the proteins of liver, plasma, and other internal organs metabolize 8.3 gm. (6.2 + 2.1) of nitrogen per day.

The remaining protein synthesis (equivalent to 15.3 − 8.3 = 7.0 gm. of nitrogen per day) must take place in the other tissues, i.e., lung, brain, bone, skin, but chiefly in muscle. Since the total nitrogen of these tissues is 1750 − 150 = 1600 gm., and 7.0 gm. are turned over per day, k (the fraction of protein degraded and resynthesized per day) is equal to 7.0/1600 = 0.0044 and $t_{\frac{1}{2}}$ is therefore 158 days. It has previously been suggested that not all constituents of muscle of the rat have equal metabolic activities. Indeed it has been possible to fractionate the muscle proteins of rats fed labeled glycine into fractions of differing isotope concentrations (8). Let us assume that in the human the tissues having a $t_{\frac{1}{2}}$ of 158 days are also metabolically heterogeneous and consist of two fractions: one of them, α, is metabolically active, having a turnover time equal to that of liver tissue ($t_{\frac{1}{2}} = 10$ days), and the other fraction, $1 - \alpha$, is metabolically

nearly inactive, with an almost infinite turnover time ($t_{\frac{1}{2}} = \infty$). The metabolically active fraction, α, can be calculated as follows.

If k_α and k_i are the fractions of the active and inactive tissues turned over per day then

$$k_\alpha(\alpha)(1600) + k_i(1 - \alpha)(1600) = 7 0 \qquad (20)$$

Since we have assumed $t_{\frac{1}{2}}$ for the active and inactive components to be 10 days and infinite respectively, then k_α and k_i are, from Equation 19, 0.0693 and 0 respectively. By insertion of these values in Equation 20 a value of 0.063 for α is obtained. On the above assumption only about 6.3 per cent of the tissue proteins other than those of liver, plasma, and internal

<div align="center">TABLE V</div>

Rate of Protein Synthesis and Size of Nitrogen Pool in Rats on Diets of Various Nitrogen Content

Experiment No.	Weight	Casein content of diet	k^*	Glycine admin-istered	λ_E / λ_0 [†] 0-24 hrs.	0-48 hrs	A^*	B^*	S^*	P^*	E_k^*	S_k^*	P_k^*
	gm.	per cent	gm.	mg.				gm.	gm.	gm.	gm.	gm.	gm.
S-31	250	0	0.080	25	0.13	0.17	0.20	0.91	0.32	0.44	0.32	1.28	1.76
S-32	318	0	0.095	25	0.12	0.17	0.20	0.91	0.38	0.52	0.30	1.20	1.64
S-14‡	270	15	0.305	35	0.39	0.48	0.56	1.07	0.24	0.51	1.13	0.89	1.89
S-15‡	290	50	1.06	50	0.65	0.74	0.81	1.35	0.25	0.97	3.66	0.86	3.35
S-41	318	81	1.60	25	0.64	0.80	0.87	1.3	0.24	1.42	5.03	0.76	4.47
S-42	314	81	1.68	25	0.60	0.75	0.83	1.2	0.34	1.70	5.35	1.08	5.42

* E = total nitrogen excretion per 24 hours; S = protein synthesized per 24 hours; P = nitrogen in metabolic pool. E_k, S_k, and P_k are the same quantities per kilo of body weight. See the text for method of evaluation of constants A and B.

† λ_E = N^{15} excreted; λ_0 = N^{15} fed = 4.36 microequivalents per mg. of glycine.

‡ Average of two rats.

organs is actively involved in the dynamic state. It is of course unlikely that only two fractions exist; it is more probable that a whole spectrum of activities occurs. More data than are here available are necessary for a further analysis of this problem.

In the rat the values of S_k obtained on diets of varying nitrogen content ranged from 0.76 to 1.28 (see Table V), with an average value of 1.01. If we assume the nitrogen content per kilo of rat tissue to be 25 gm. and use S_k equal to 1.01, then k (the fraction of protein degraded and resynthesized per day) is 1.01/25.0 = 0.04. By substitution of this value in Equation 19 a value of $t_{\frac{1}{2}} = 17$ days is obtained. In the rat, the value of $t_{\frac{1}{2}}$ for the proteins of liver, plasma, and internal organs is close to 6 days (8). The fraction of these proteins regenerated per day is, therefore, $k = 0.693/t_{\frac{1}{2}} = 0.693/6 = 0.12$. The proteins of liver, plasma, and internal organs contain

about 2.2 gm. of nitrogen per kilo of body weight.[3] Thus $0.12 \times 2.2 = 0.26$ gm. of nitrogen per kilo is employed daily by these tissues for the synthesis of protein. The remainder, $1.01 - 0.26$, or approximately 0.75 gm. of nitrogen, must be accounted for by protein synthesis in the carcass (muscle, skeleton, lung, brain, and skin). Since $25.0 - 2.2 = 22.8$ gm. of nitrogen per kilo of body weight is in carcass protein, the fraction regenerated per day is $k = 0.75/22.8 = 0.033$. The half life time of carcass proteins is $t_{\frac{1}{2}} = 0.693/0.033 = 21$ days.

If, as for the case of the human, we assume that carcass protein consists of two fractions, one of which, α, has a turnover time equal to that of liver proteins ($t_{\frac{1}{2}} = 6$ days) and the other $(1 - \alpha)$ has a turnover time of nearly infinity ($t_{\frac{1}{2}} = \infty$), then from Equation 20

$$22.8 \alpha k_\alpha + 22.8(1 - \alpha)k_i = 0.75$$

where k_α and k_i are equal to 0.12 and 0 respectively; and

$$\frac{0.75}{22.8 \times 0.12} = 0.28$$

On this crude assumption, about 28 per cent of carcass protein is involved in the dynamic state and 72 per cent is unreactive.

It is apparent that the rat and the human are quite different in their rates of protein rejuvenation. The original observations of the extreme instability of the cellular proteins (1), which gave rise to the concept of the dynamic state, are more typical of the rat ($t_{\frac{1}{2}} = 17$ days) than of the human ($t_{\frac{1}{2}} = 80$ days). While the proteins of the internal organs, both in the rat and the human, are extensively involved in the dynamic state, this appears to be far less so for the muscle proteins. A large fraction of the muscle proteins of the rat and an even larger fraction in man seem to be relatively metabolically inert.

The approach to problems of protein turnover exemplified in this study offers a sounder basis than has heretofore been available for the study of nutritional and endocrine relationships. By the use of these methods it is possible to assess a dietary deficiency (e.g., an amino acid or vitamin deprivation) or an endocrine disturbance in terms of its direct effect on the rate of protein synthesis and the size of the nitrogen pool.

EXPERIMENTAL

Glycine labeled with 32.9 atom per cent excess N^{15} was synthesized from isotopic ammonia (11).

The isotopic glycine was given to the human subjects by mouth in a single dose at a level of 10 mg. per kilo of body weight. It was adminis-

---

[3] Approximately this value has been found in our laboratory (1, 5, 8–11).

tered to rats by stomach tube at a level of 80 to 170 mg. per kilo of body weight.

Urine specimens were collected at various intervals and analyzed for total nitrogen and N^{15}. From the data the fraction of N^{15} excreted was calculated (see Tables I, II, III, and V). The N^{15} concentration of the urine was determined by the method described in the preceding paper (12). Urea nitrogen was obtained as ammonia by treating diluted urine, from which ammonia had been adsorbed with permutit, with urease.

The rats were given diets of the following percentage composition: low protein diet, starch 83, brewers' yeast 5, salt mixture (13) 4, cottonseed oil (Wesson oil) 6, cod liver oil 2. The 15 and 50 per cent casein diets were prepared by replacing starch with casein. In the high protein diet the starch was replaced by 81 per cent casein and 2 per cent Celluration (Eimer and Amend).

SUMMARY

The rate of interaction of N^{15}-labeled glycine with the body proteins has been measured in rats and human subjects. From these data it is possible to calculate the rate of protein synthesis and the size of the nitrogen pool. The rates of protein synthesis per kilo of body weight are 1.0 and 0.20 gm. of nitrogen per day for the rat and human respectively.

From the approximately known turnover times of the different organs and the synthetic rates for the whole organism, it appears that only a fraction of the muscle proteins is extensively involved in the dynamic state.

BIBLIOGRAPHY

1. Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **130**, 703 (1939).
2. Rittenberg, D., and Schoenheimer, R., *Physiol. Rev.*, **20**, 218 (1940).
3. Bloch, K., *J. Biol. Chem.*, **165**, 469 (1946).
4. Bloch, K., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **138**, 155 (1941).
5. Ratner, S., Rittenberg, D., Keston, A. S., and Schoenheimer, R., *J. Biol. Chem.*, **134**, 665 (1940).
6. Vierordt, H., Anatomische, physiologische, und physikalische Daten und Tabellen, Jena (1906).
7. Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M., *J. Biol. Chem.*, **144**, 541 (1942).
8. Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **153**, 401 (1944).
9. Ratner, S., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **134**, 653 (1940).
10. Tesar, C., and Rittenberg, D., *J. Biol. Chem.*, **170**, 35 (1947).
11. Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127**, 301 (1939).
12. Sprinson, D. B., and Rittenberg, D., *J. Biol. Chem.*, **180**, 707 (1949).
13. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).

STUDIES ON THE DESOXYPENTOSE NUCLEASE OF YEAST AND ITS SPECIFIC CELLULAR REGULATION*

By STEPHEN ZAMENHOF† AND ERWIN CHARGAFF

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

Although the enzymatic cleavage of the desoxypentose nucleic acid macromolecules of the cell nucleus probably is an important biological phenomenon, nothing is known about the mechanisms which regulate it within the cell. While such a regulatory mechanism does not appear to have been postulated previously, its existence may be inferred from certain experimental observations. For instance, the agent inducing the transformation of pneumococcal types is regarded as a specific desoxypentose nucleic acid (1) that is extremely sensitive to the action of depolymerizing enzymes; it is destroyed on contact with minute amounts of pancreatic desoxyribonuclease (2) or with a similar enzyme present in the pneumococcus itself (3). Yet, this nucleic acid is able to exert its transforming effect on the living cell.

It is obvious that if cells possess controlling devices for the growth and the cleavage of the desoxypentose nucleic acid molecules, several different types of control could be operative. The present study, some of the results of which have been reported in a brief communication (4), deals with one instance, namely the regulatory mechanism of the yeast cell.

EXPERIMENTAL[1]

Measurement of Enzyme Activity

Substrate—A highly polymerized preparation of DNA from calf thymus (see (5), Preparation 3) served as the substrate. A standard solution in veronal buffer of the appropriate pH contained 2.4 mg. of DNA and 5 μM of magnesium per cc. In some experiments a DNA preparation from yeast (6) also was employed.

Methods of Measurement—DNase activity was measured by following the drop in viscosity of the substrate solution, incubated with the enzyme

[1] For the sake of brevity, desoxypentose nucleic acid will be referred to as DNA, desoxypentose nuclease as DNase.

at 30° in the viscosimeter. A micro viscosimeter (Otto R. Greiner Company, Newark, New Jersey) of the Ostwald type was employed (water value 19 seconds), which permitted the use of as little as 0.35 cc. of fluid. The mixture of 0.25 cc. of substrate and 0.1 cc. of enzyme (both prewarmed to 30°) was introduced into the viscosimeter by means of a capillary pipette and the viscosity determined at frequent intervals. These intervals were recorded as the time elapsed between the addition of the enzyme (zero time) and the moment when, in each viscosity measurement, the liquid had reached the half way point between the viscosimeter marks. When, as was the case with active enzyme preparations, high enzyme dilutions had to be employed, the solutions were stabilized by the addition of gelatin (7) which was present in a final concentration of 0.01 per cent in the enzyme-substrate mixtures.

Unit of Enzyme Activity—The DNase unit defined by McCarty (7) was adopted in the present work. In order to correct for differences in the viscosimeters used, a purified preparation of pancreatic desoxyribonuclease[2] (10,000 units per mg. of protein) (7) was used as a standard in all determinations.

The protein content of the DNase preparations was estimated by a modification of the method of Robinson and Hogden (8).

Measurement of Inhibitor Activity

The activity of the DNase inhibitor preparations was expressed as per cent of DNase inhibition. To 0.25 cc. of the substrate solution 0.1 cc. of a mixture of equal volumes of enzyme and inhibitor solutions was added and the viscosity changes were recorded as described before. Controls in which the inhibitor solution was replaced by water were run simultaneously.

Preparation of Yeast Desoxypentose Nuclease

The extract with M sodium chloride solution of the cell débris prepared from ground yeast includes, as shown in a preceding paper from this laboratory (6), a highly polymerized DNA. The same extract also contains a DNase in a largely inhibited state from which it is released gradually on storage at 4°. Aqueous extracts of the crushed cells exhibited practically no DNase activity, nor could the latter be detected in autolysates of intact yeast.

In a typical preparation, 350 gm. of fresh bakers' yeast (*Saccharomyces cerevisiae*) were washed with water and the centrifuged cells were suspended in 60 cc. of water and crushed in the bacterial mill (6). Following this

operation, the mixture was centrifuged for 2 hours at 1900 × g (4000 R.P.M.). The opalescent supernatant (135 cc.) served for the preparation of the inhibitor described later. The débris layer was suspended in 300 cc. of M sodium chloride solution and the viscous mixture (500 cc.) kept at 4° for 4 months, during which period the viscosity disappeared completely. Repeated assays for DNase activity showed the extract to contain only 0.6 unit per cc. during the first 2 weeks of storage; after 19 days it had 17 units, after 52 days 25 units, and after 90 to 120 days 30 units per cc.

The supernatant resulting from the centrifugation of the mixture at 1900 × g for 1 hour was subjected to a rocking dialysis against ice-cold distilled water for 4 hours and then evaporated in the frozen state in a vacuum. The solution of the evaporation residue, which still contained salt, in 30 cc. of water was clarified by centrifugation at 31,000 × g for 2 hours and ammonium sulfate was added to the yellow supernatant to bring it to 0.6 saturation. The resulting precipitate was collected by centrifugation at 31,000 × g and drained by suction (Whatman filter paper, No. 50). Its suspension in 45 cc. of water was dialyzed, with rocking, against ice-cold distilled water for 7 hours and centrifuged at 31,000 × g for 1 hour. The sediment was washed with water and then extracted with 30 cc. and again with 12 cc. of M sodium chloride solution. The combined extracts were clarified (31,000 × g, 1 hour), dialyzed (6 hours), and dried in a vacuum from the frozen state. The DNase preparation weighed 27 mg. and formed a slightly yellowish fluff that was insoluble in distilled water and soluble in salt solutions. This experiment is summarized in Table I.

Preparation of Crude Yeast Protease

In order to test the assumption that the gradual activation of yeast DNase on storage was due to the removal of an inhibitor of protein nature, it was found advisable to examine, among other enzymes, a proteolytic enzyme preparation derived from yeast itself. Procedures described in the literature (9) were not successful in our hands, but a crude preparation could be obtained in the following manner. A fresh suspension of the débris layer from crushed yeast cells in M sodium chloride solution was prepared as described before, and 70 cc. of the mixture were kept at 4° for 3 weeks. The supernatant collected after centrifugation at 1900 × g for 90 minutes was subjected to a rocking dialysis against ice-cold distilled water for 6 hours. The solution (protected by 0.002 per cent ethyl mercurithiosalicylate) was kept at 26° for 15 hours in order to destroy DNase, and then evaporated from the frozen state in a vacuum. The dry preparation weighed 880 mg. and was tested on gelatin by the method of Northrop and Hussey

(10). This material in a final concentration of 6 mg. per cc. at pH 5 reduced the viscosity of the gelatin solution by 5 per cent in 186 seconds.

Crude Desoxypentose Nuclease from Various Sources

Neurospora crassa was grown on Difco malt extract broth. The washed mycelium (dry weight 3.7 gm.) was crushed in 140 cc. of water by means of a high speed mixer. Following centrifugation for 1 hour at 1900 $\times$ g, the supernatant was subjected to a rocking dialysis against ice-cold distilled water for 6 hours and evaporated in the frozen state *in vacuo*. The powder (1.4 gm.) contained 63 DNase units per mg. of solid.

TABLE I

Preparation of Yeast Desoxypentose Nuclease

Step No.	Fraction	Amount	Total activity	Activity per mg. solid	Activity per mg. protein
			units	*units*	*units*
1	Yeast cake, washed and crushed; débris layer extracted with M NaCl	350 gm.			
2a	M NaCl extract, fresh	500 cc.	300		
2b	" " " after 4 mos. at 4°	500 "	15,200		
3	Activated extract, dialyzed and dried	15 gm.	15,000	1	14
4	Solution of dry powder precipitated at 0.6 saturation with $(NH_4)_2SO_4$; ppt. redissolved and dialyzed	57 cc.	10,200		
5a	Dialyzed mixture centrifuged; supernatant (discarded)	56 "	420		
5b	Sediment washed, extracted with M NaCl, dialyzed	51 "	6,200		
6	Dialyzed mixture, evaporation residue	27 mg.	4,300	160	1250

A powerful malt DNase was obtained by the aqueous extraction of 4 day-old germinating barley seeds ground with glass powder, or, more conveniently, from commercial malt diastase (Eimer and Amend, New York). An extract of 10 gm. of the product with 50 cc. of water was clarified by centrifugation, dialyzed for 7 hours against ice-cold distilled water, and again centrifuged. The supernatant, evaporated in the usual fashion, yielded 350 mg. of a substance having 300 DNase units per mg. of solid.

▶ Even with calf thymus, indications of the existence of a DNase were obtained. 1 kilo of the fresh tissue was cleaned, washed, and ground in a high speed mixer with about 1 volume of water. The fresh mixture ex-

hibited no DNase activity. The mixture (containing 0.002 per cent ethyl mercurithiosalicylate) then was kept at 4° for 3 weeks and centrifuged at 1900 × *g* for 1 hour. The clear supernatant (1270 cc.) was brought to pH 4.5 by means of 0.25 N sulfuric acid and to 0.2 saturation with solid ammonium sulfate, filtered through infusorial earth, and the filtrate was adjusted to 0.8 $(NH_4)_2SO_4$ saturation. The sediment, removed by centrifugation at 1900 × *g* for 1 hour, was well drained on filter paper with suction and dissolved in 50 cc. of water. The solution was dialyzed for 7 hours against ice-cold distilled water, clarified by centrifugation, frozen, and evaporated. The almost white, fluffy powder (3.3 gm.) had a weak, but discernible DNase activity, 0.5 unit per mg. of solid.

Preparation of DNase Inhibitor from Yeast

As mentioned in a preceding section dealing with the preparation of yeast DNase, the yeast cells (350 gm.) were crushed by passing their suspension in water through the bacterial mill. Centrifugation of the mixture yielded an opalescent supernatant (135 cc.) which was filtered through infusorial earth. The almost clear, yellow filtrate was brought to 0.84 saturation with solid ammonium sulfate. Following centrifugation for 1 hour at 1900 × *g*, the solution of the drained sediment in 42 cc. of water was dialyzed with rocking against ice-cold distilled water for 7 hours, filtered through infusorial earth, frozen, and evaporated in a vacuum, when 1.2 gm. of loose white flakes were obtained. All operations were performed in the cold and within 2 days.

Properties of Purified Yeast Desoxypentose Nuclease

Activity—The purified yeast DNase had, as shown in Table I, an activity of 1250 units per mg. of protein. It produced, even in high dilutions (less than 2 γ of protein per cc.), a rapid drop in the viscosity of thymus DNA (Curve A, Fig. 1). No proteolytic, desoxypentose nucleotidase, or ribonuclease activities were demonstrable in the yeast DNase when as much as 80 γ of protein per cc. was employed.[*]

It appeared, in this connection, of interest to compare the depolymerizing effects of yeast DNase on the DNA from its own species (yeast) and on the DNA from a foreign source (calf thymus). The results are illustrated in Fig. 2, which shows the absence of species specificity in this respect; both nucleic acids were depolymerized at about the same rate.

[*] The yeast DNase was assayed for proteolytic activity by the previously mentioned method (10). The failure to produce inorganic phosphate from thymus DNA and dialyzable fragments from yeast ribonucleic acid indicated the absence of nucleotidase and ribonuclease respectively.

Comparison with Pancreas DNase—In some respects, the yeast DNase resembled the DNase of pancreas (7, 11). It required, for instance, magnesium ion for activation and it was labile to heat; the activity was completely destroyed when the enzyme solution was kept at 55° for 15 minutes. But there were several important features which distinguished it from the pancreatic enzyme. It was insoluble in water, but soluble in
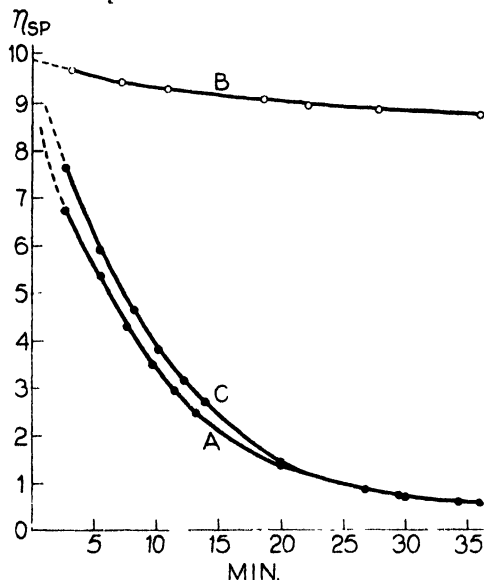
FIG. 1. The specific viscosities of mixtures of thymus DNA (sodium salt) and yeast DNase (with and without inhibitor) are plotted as the ordinate. The abscissa indicates the duration of incubation of the mixtures before testing (both at 30°). For Curve A the mixture contained 7.4 mg. of DNA, 10.5 μM of Mg, 8 units of yeast DNase, 1 cc. of water, and 2.5 cc. of veronal buffer (pH 6.6); for Curve B, the water was replaced by 1 cc. of the supernatant (31,000 $\times$ g) of a fresh aqueous extract of crushed yeast (derived from 300 mg. of crushed cells); for Curve C by 1 cc. of an aqueous suspension of the sediment from centrifugation at 31,000 $\times$ g.

salt solutions. This may be deduced even from the method of preparation of the enzyme (Table I, Steps 5a and 5b).

Another distinction between the two enzymes becomes evident from a comparison of their pH optima as it is provided in Fig. 3. The activity optimum of the yeast DNase was found in the vicinity of pH 6; at pH 8.1 only 20 per cent of the activity was retained. The pancreas DNase, on the other hand, exhibited its maximum activity around pH 7.4 (*cf.* also McCarty (7)); more than 75 per cent of the activity was preserved at pH 8.1, but less than 20 per cent at pH 6.

The most significant difference between the two enzymes arose, however, from the discovery in yeast of a specific inhibitor of the yeast DNase which
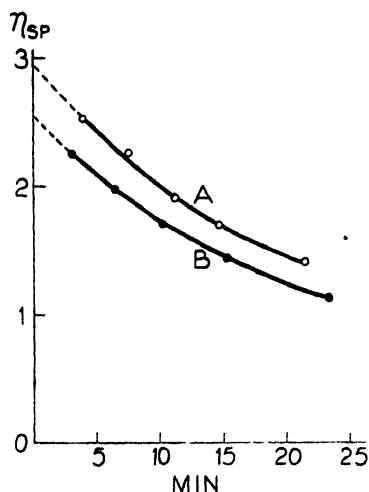
FIG. 2. The specific viscosities of mixtures of yeast DNase with solutions of yeast DNA (Curve A) and calf thymus DNA (Curve B) are plotted as the ordinate; abscissa as in Fig. 1. The mixtures contained, in addition to 2 units of yeast DNase and 3 μM of Mg per cc. of veronal buffer (pH 6.6), 1.2 mg. of yeast DNA (Curve A) or 0.9 mg. of calf thymus DNA (Curve B).
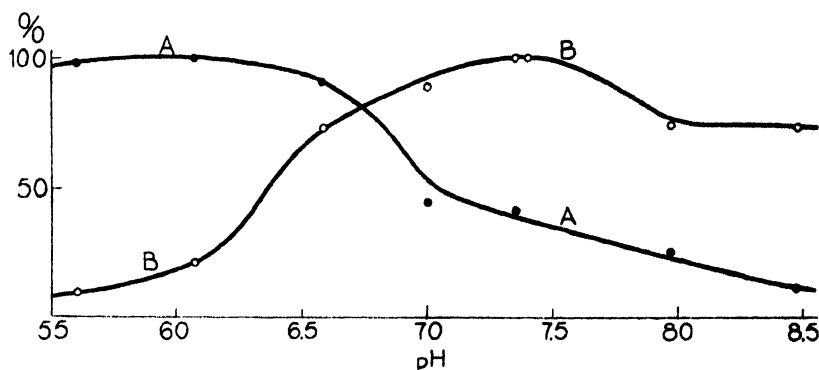
FIG. 3. The activities of yeast DNase (Curve A) and pancreatic DNase (Curve B) are plotted, in per cent of maximum activity, as the ordinate. The abscissa indicates the pH of the mixtures of substrate, enzyme, and buffer. All other conditions were the same as for Curve A in Fig. 1.

failed to act on the pancreatic enzyme. This inhibitor is discussed in the following section.

Properties of DNase Inhibitor from Yeast

Activity—The DNase inhibitor present in aqueous extracts of crushed yeast cells caused up to 94 per cent inhibition of the yeast DNase activity. A typical experiment is reproduced as Curves A and B of Fig. 1.

The inhibitor did not suppress the activities of purified pancreas DNase (7) and of the crude DNase preparations, described before, that were derived from *Neurospora crassa*, germinating barley, and calf thymus. When the yeast inhibitor was added to a mixture of the DNases from yeast and pancreas, the combined enzymatic activity dropped to the level of the pancreas DNase alone, thus indicating that of the mixture of DNases only the yeast DNase activity had been suppressed (Fig. 4).

Properties—A comparison of Curves B and C in Fig. 1 will show that the entire inhibitory activity of fresh aqueous extracts of crushed yeast cells remained in the supernatant after centrifugation at 31,000 $\times$ *g* for 150 minutes. The lipoprotein pellet, sedimenting at this centrifugal speed, was devoid of inhibitor activity.

The inhibitor was labile to heat; less than 5 minutes at 55° was sufficient to destroy the activity. It was inactivated by crystalline trypsin, by ficin (Merck), and by the proteolytic enzyme from yeast previously described.

These inactivation experiments were conducted by incubating 2 cc. of the inhibitor solution (either as the fresh aqueous extract of crushed yeast or as a 1.5 per cent solution in water of the dry preparation of the inhibitor previously discussed) at 30° with 0.25 cc. of solutions of the various proteolytic enzymes. The incubation periods required for the complete inactivation of the DNase inhibitor were the following (the protease concentration in the final incubation mixture given in parentheses): ficin (6.7 mg. per cc.), 3 to $4\frac{1}{4}$ hours; crystalline trypsin (0.5 mg. per cc.), $3\frac{1}{2}$ to $5\frac{1}{4}$ hours; yeast protease (13 mg. per cc.), $5\frac{1}{4}$ to 6 hours; control (without proteolytic enzyme), $16\frac{1}{2}$ to 20 hours. In order to test for the survival of the inhibitor, a portion of the incubation mixture was, after various intervals, added to an equal volume of fresh yeast DNase (8 units per cc.) and the DNase activity determined as described above. In the experiments with trypsin it was found expedient to inactivate the proteolytic enzyme by the addition of an excess of the crystalline trypsin inhibitor from soy bean (12)[4] before testing the mixture on DNase, so as to protect the latter enzyme from tryptic digestion. An adequate number of controls was run to ascertain that neither the proteolytic enzymes nor the soy bean inhibitor had any DNase or DNase inhibitor activity.

The DNase inhibitor, therefore, appears to be a water-soluble, rather labile protein, precipitable with ammonium sulfate at 0.84 saturation.

---

[4] We are greatly indebted to Dr. M. Kunitz for this preparation.

The inactivation of inhibitor solutions on storage at 30° for about 20 hours or at 4° within 2 to 4 weeks probably is attributable to the presence of the yeast protease mentioned before.
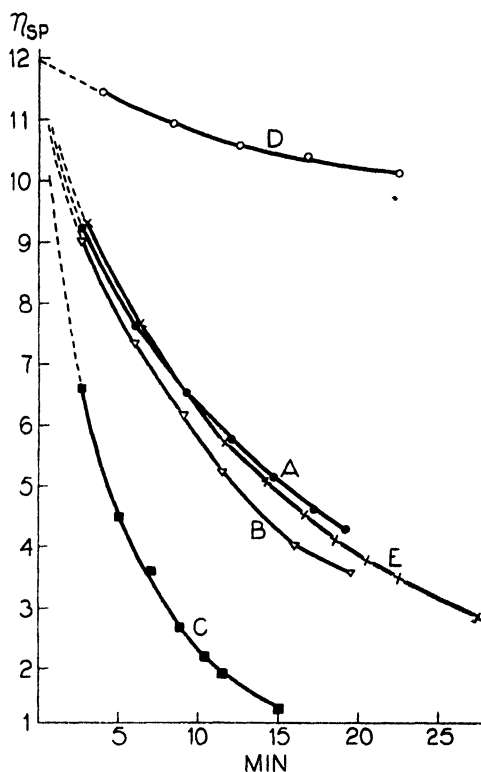
Fig. 4. The specific viscosities of the mixtures are plotted as the ordinate; abscissa as in Fig. 1. All mixtures contained 1.7 mg. of calf thymus DNA and 3 μM of Mg per cc. of veronal buffer (pH 6.7). In addition, the mixture for Curve A contained 1.8 units of yeast DNase; for Curve B, 2 units of pancreas DNase with or without 3 mg. of yeast inhibitor; for Curve C, 2 units of pancreas DNase and 1.8 units of yeast DNase; for Curve D, 1.8 units of yeast DNase and 3 mg. of yeast inhibitor; for Curve E, 2 units of pancreas DNase, 1.8 units of yeast DNase, and 3 mg. of yeast inhibitor.

Nature of Inhibition Reaction—The inhibition reaction appears to be instantaneous. The relationship between the amount of DNase inhibitor employed and the extent of inhibition is illustrated in Fig. 5. These experiments suggest that the inhibition reaction is of the reversible type, similar to the inactivation of pepsin by the pepsin inhibitor ((13) p. 90) or of chymotrypsin by the soy bean inhibitor ((13) p. 157). The diagram

shows that with increasing inhibitor concentrations the percentage of inhibition asymptotically approaches a value somewhat below 100 per cent. It is likely that, even with large amounts of inhibitor, no complete inhibition could be attained. This agrees with the finding reported before that fresh M NaCl extracts of crushed yeast exhibited a slight DNase activity, about 2 per cent of the total obtainable activity (Step 2a, Table I).

The relationship between inhibition and substrate concentration also was studied. When the action of the inhibitor was expressed as the ratio $V:V_i$, where V is the velocity of the viscosity drop for the non-inhibited DNase and V_i that for the 87 per cent inhibited enzyme, a practically con-
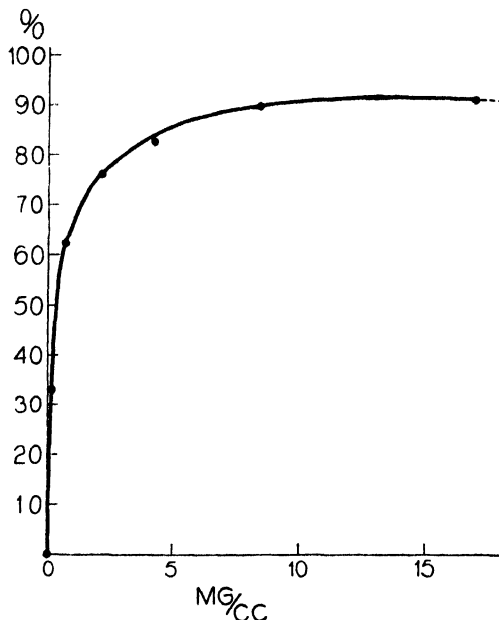
Fig. 5. The percentage of inhibition of yeast DNase (ordinate) is plotted against the inhibitor concentration (abscissa). Other experimental conditions as in Fig. 1, except that the solid inhibitor preparation was used.

stant ratio was found over a range of substrate concentrations (thymus DNA) from 0.85 to 1.75 mg. per cc. This suggests that the inhibition is non-competitive.

DISCUSSION

The purpose of the present study is not only to add one more instance of the occurrence of enzymes able to degrade highly polymerized desoxypentose nucleic acids, but to provide a clue, even though incomplete and rudimentary, to the mechanisms by which these enzymes are controlled

in the cell. To gain an insight into the ways in which the cell maintains its own continuity and protects itself against agents attempting to transform it will require an understanding of many systems, of which the present example, perhaps, is one.

A study of the enzymes through which the cell achieves the breakdown of the large molecules of desoxypentose nucleic acid offers, in this connection, much interest. The existence of such enzymes has been known since the work of Araki (14). They occur in a variety of mammalian tissue extracts (14–16), in eggs (17), in plants (15, 18), and in microorganisms (3, 19–21). It is, in fact, likely that they are ubiquitous cell constituents, either in a free or in a masked form. Many of these reports dealt with crude extracts or bacterial cultures that required relatively long incubation periods, in order to reveal the enzyme. But one mammalian depolymerase, the pancreatic desoxyribonuclease, has been highly purified (7) and obtained in crystalline form (11). A recent note from this laboratory reported on the products of its action on the desoxyribonucleic acid of calf thymus (22).

Whether the depolymerase present in large quantities in pancreatic juice, whose function probably is primarily digestive, may be compared with the intracellular enzymes of the type discussed here cannot yet be stated. It is not unlikely that the latter represent a functionally and chemically distinct group of enzymes, possibly endowed with a high degree of specificity. It was, in fact, this question of specificity that led to the investigations reported here, though the results were unexpected.

The possibility of the existence of specific chemical differences between the macromolecular desoxypentose nucleic acids of different cellular origin has been discussed elsewhere (23, 24). That very remarkable differences do exist was recently shown in this laboratory (5, 25). Such divergence, whether with respect to proportion, sequence, or nature of the component nucleotides, is in harmony with the hypothesis connecting nucleic acids with the transmission of hereditary characteristics. The transformation experiments of Avery and his associates were cited at the beginning of this paper (1–3). Another impressive example was provided recently by Boivin et al. (26) who showed that the absolute quantity of desoxypentose nucleic acid was constant per bovine nucleus, regardless of the tissue from which it came, with the exception of spermatozoa which contained about one-half.

If, as is probable, the highly polymerized desoxypentose nucleic acids are an essential part of the hereditary processes, their duplication may be assumed to be a necessary, perhaps even the first, step preparatory to the division of the cell. Since, however, the synthetic mechanisms by which the cell achieves the formation of the nucleic acid macromolecules are as

yet unknown, it cannot be said whether degradation, as studied here, and synthesis proceed by similar routes. But it is, in any event, probable that the enzymatic depolymerization represents the first catabolic step. It has been shown that phosphatases, whose occurrence in the nucleus is indicated by histochemical evidence (27–29), are unable to attack desoxypentose nucleic acid before its depolymerization to smaller fragments (28, 30, 31).

The experiments presented here have demonstrated the existence in yeast cells of a desoxypentose nuclease that is found in a largely inhibited state in fresh salt extracts of the crushed cells. Two points deserve mention in this connection. The desoxypentose nuclease of yeast apparently never is completely inactive in the cell, nor can it be entirely inactivated even by large amounts of the inhibitor (Fig. 5). The cell fraction that contained the desoxyribonucleic acid of yeast (6), namely the débris layer, also was found to contain practically the entire amount of depolymerizing enzyme. This finding is in agreement with the assumption that the nucleus is the probable site of the depolymerizing enzyme (17).

The present studies have, furthermore, provided indications that yeast desoxypentose nuclease differs, in many important respects, from the desoxyribonuclease of pancreas. Its solubility and pH optimum are different and, more significantly, it is specifically inhibited by an agent found in yeast cells. This inhibitor, a water-soluble, heat-labile protein whose action on the yeast depolymerase was instantaneous, non-competitive, and reversible, failed to suppress the activity of depolymerizing enzymes from other sources, *viz.*, pancreas, thymus, germinating barley, and even *Neurospora*. The yeast enzyme depolymerized the desoxyribonucleic acids of yeast and of thymus at about the same rate (Fig. 2). Since these compounds have been shown to differ significantly with regard to their purine and pyrimidine composition (5, 25), the measurement of the viscosity drop apparently is not a tool sufficiently sensitive to reveal specific chemical differences between nucleic acids derived from different species.

The problem of intracellular desoxypentose nucleases is closely connected with the problem of the regulation of their action within the cell. Desoxypentose nucleic acids appear to occur in the cell in a highly polymerized form; there are indications that depolymerization results in the loss of biological activity (2). The cell must, therefore, be endowed with a regulatory system to prevent a rapid depolymerization to the non-functional stage. Such controls could be conceived as being mediated by a number of mechanisms, *e.g.*, the existence of a specific inhibitor, as postulated here, the maintenance within the cell of conditions (*e.g.*, pH) detrimental to the enzymatic reaction, and the spatial separation within the cell of enzyme and substrate.

On the basis of the work presented here, the following sequence of autolytic reactions, possibly held in a delicate balance in the living yeast cell, could be formulated: (a) activation of yeast protease (9); (b) digestion of the desoxypentose nuclease inhibitor; (c) liberation of active desoxypentose nuclease; (d) depolymerization of desoxypentose nucleic acid. It would be inviting to assume that such relations between specific inhibitor and enzyme, in some ways reminiscent of immunological reactions, are of more general biological occurrence; however, much more work will have to be done before this can be said. It may very well be that this is a case in which the universality of a phenomenon is obscured by the multiplicity of its appearances.

SUMMARY

The isolation from yeast cells of a desoxypentose nuclease (DNase) and of its specific inhibitor is described. The fresh m NaCl extract of crushed yeast contains the enzyme in a largely inhibited state. Upon prolonged storage of the extract in the cold, the active DNase is released, owing to the destruction by yeast protease of the inhibitor. The yeast DNase inhibitor specifically inhibits yeast DNase; it does not affect the DNase from other sources, viz. pancreas, thymus, germinating barley, *Neurospora crassa*. It is shown that yeast DNase differs in many respects from the pancreatic enzyme. The preparation and properties of the various yeast fractions taking part in these reactions are described and some of the biological implications of these findings are discussed.

BIBLIOGRAPHY

1. Avery, O. T., MacLeod, C. M., and McCarty, M., *J. Exp. Med.*, **79**, 137 (1944).
2. McCarty, M., and Avery, O. T., *J. Exp. Med.*, **83**, 89 (1946).
3. McCarty, M., and Avery, O. T., *J. Exp. Med.*, **83**, 97 (1946).
4. Zamenhof, S., and Chargaff, E., *Science*, **108**, 628 (1948).
5. Chargaff, E., Vischer, E., Doniger, R., Green, C., and Misani, F., *J. Biol. Chem.*, **177**, 405 (1949).
6. Chargaff, E., and Zamenhof, S., *J. Biol. Chem.*, **173**, 327 (1948).
7. McCarty, M., *J. Gen. Physiol.*, **29**, 123 (1946).
8. Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **135**, 707, 727 (1940).
9. Grassmann, W., and Dyckerhoff, H., *Z. physiol. Chem.*, **179**, 41 (1928).
10. Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, **5**, 353 (1923).
11. Kunitz, M., *Science*, **108**, 19 (1948).
12. Kunitz, M., *J. Gen. Physiol.*, **29**, 149 (1946).
13. Northrop, J. H., Kunitz, M., and Herriott, R. M., Crystalline enzymes, New York, 2nd edition (1948).
14. Araki, T., *Z. physiol. Chem.*, **38**, 84 (1903).
15. de la Blanchardière, P., *Z. physiol. Chem.*, **87**, 291 (1913).
16. Greenstein, J. P., and Jenrette, W. V., *Cold Spring Harbor Symposia Quant. Biol.*, **9**, 236 (1941).

17. Mazia, D., *Cold Spring Harbor Symposia Quant. Biol.*, **9**, 40 (1941).
18. Greenstein, J. P., *Federation Proc.*, **1**, 113 (1942).
19. Plenge, H., *Z. physiol. Chem.*, **39**, 190 (1903).
20. Cohen, S. S., *J. Biol. Chem.*, **168**, 511 (1947).
21. Tillett, W. S., Sherry, S., and Christensen, L. R., *Proc. Soc. Exp. Biol. and Med.*, **68**, 184 (1948).
22. Zamenhof, S., and Chargaff, E., *J. Biol. Chem.*, **178**, 531 (1949).
23. Chargaff, E., *Cold Spring Harbor Symposia Quant. Biol.*, **12**, 28 (1947).
24. Chargaff, E., and Vischer, E., *Ann. Rev. Biochem.*, **17**, 201 (1948).
25. Vischer, E., Zamenhof, S., and Chargaff, E., *J. Biol. Chem.*, **177**, 429 (1949).
26. Boivin, A., Vendrely, R., and Vendrely, C., *Compt. rend. Acad.*, **226**, 1061 (1948).
27. Danielli, J. F., and Catcheside, D. G., *Nature*, **156**, 294 (1945).
28. Krugelis, E. J., *Genetics*, **31**, 221 (1946).
29. Nickerson, W. J., Krugelis, E. J., and Andrèsen, N., *Nature*, **162**, 192 (1948).
30. Schmidt, G., and Levene. P. A., *Science*, **88**, 172 (1938).
31. Schmidt, G., Pickels, E. G., and Levene, P. A., *J. Biol. Chem.*, **127**, 251 (1939).

ON THE LIPOPROTEIN PARTICLES OF YEAST CELLS*

By MELVIN A. NYMAN† AND ERWIN CHARGAFF

(*From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York*)

In connection with studies on macromolecular lipoproteins in which this laboratory has been engaged for some time, it appeared of interest to examine a representative of this class of compounds derived from a microbial source. For this purpose *Saccharomyces cerevisiae* was chosen. The preparation of the starting material, crushed yeast cells, followed methods previously employed (1, 2); the procedures for the isolation of the lipoprotein were essentially similar to those used for the preparation of the thromboplastic protein and other lipoproteins from animal tissue cells (3–5).

EXPERIMENTAL

Preparation[1]

In a typical experiment, 400 gm. of washed bakers' yeast were suspended in 100 cc. of 0.1 M sodium citrate solution (pH 6.9) and crushed in a bacterial mill (6), constructed by Unicam Instruments, Ltd., Cambridge, England, as described previously (1). The ground material, diluted to a light suspension with additional sodium citrate solution, was centrifuged for 45 minutes at 1900 × g. The supernatant (360 cc.) was poured through a rapid filter and the filtrate subjected to centrifugation at 31,000 × g for 105 minutes by means of the multispeed attachment of the refrigerated International centrifuge. The suspension of the sediment in 0.1 M borate buffer (pH 7.4) was centrifuged at 31,000 × g for 90 minutes and this washing was repeated twice until the supernatants were free of protein. Centrifugation of the borate buffer suspension of the pellet at 5000 × g for 20 minutes removed a small fraction of coarse aggregates which were washed once more under the same conditions.

The combined supernatants from the centrifugation at 5000 × g were subjected to dialysis against running and ice-cold distilled water for a total of 72 hours and freed of water in a vacuum in the frozen state. The resulting lipoprotein preparation is listed as Preparation 1 in Table I. The coarse aggregates that sedimented at 5000 × g, but not at 1900 × g,

[1] All operations were carried out in the cold.

were recovered in a similar fashion; they amounted to only 0.07 per cent of the starting material and were not further examined.

Two other preparations (Nos. 2 and 3) were obtained in an essentially similar way. For the isolation of Preparation 3, derived from a larger quantity of crushed yeast cells, the solution of the particles, after the removal of the débris at 1900 × g, was passed through a cooled Sharples Laboratory supercentrifuge, rotating at 50,000 R.P.M., at the rate of 16 cc. per minute. The sediment then was treated as described before.

Properties

The preparations formed almost white fluffs, easily dispersible in buffer solutions. The microscopic examination of smears treated with Feulgen's nucleal stain revealed a small number of red bodies similar to the chromosome-like structures demonstrable by the same stain in intact yeast cells.

TABLE I

Composition of Yeast Lipoprotein Particles

Preparation No.	Intact lipoprotein				Lipide-free residue		Lipides
	Yield	N	P		N	P	
	per cent	*per cent*	*per cent*	*per cent of lipoprotein*	*per cent*	*per cent*	*per cent of lipoprotein*
1	0.6	8.9	1.4	72	12.1	1.3	26
2	0.7	8.5	1.2	76	11.9	1.1	22
3	0.4	9.5	1.3	71	12.2	1.0	24

The composition of the preparations is summarized in Table I. The variation in yields is probably attributable to differences in the extent to which the cells had been crushed before extraction.

The electrophoretic properties[2] of two preparations were examined in borate buffer of pH 8.4. Preparation 1 (Table I) was almost homogeneous; a component, comprising about 95 per cent of the total, had a mobility (descending boundary) of -7.5×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$; a minor fraction (5 per cent) had the mobility of -5.4. The corresponding components of Preparation 2 had mobilities of -7.1 (about 80 per cent) and -6.1 (20 per cent). The area estimations are only approximate, since the fast moving major components were opaque even to infra-red light.

Preparation 1 was tested for thromboplastic activity in the arrangement described previously (4), but none was found. The preparations were likewise devoid of ribonuclease.

Lipides

For the extraction of lipides the lipoprotein preparations were heated for 24 hours under a reflux in a nitrogen atmosphere with a mixture of equal parts of ethanol and ether (10 cc. of liquid per 100 mg. of substance). The protein residue was removed by centrifugation, washed repeatedly with alcohol-ether, and dried *in vacuo*. The evaporation residue of the extract was dissolved in ether, shaken with aqueous 10 per cent sodium chloride, and the lipides were then recovered in the usual manner. The composition of the lipide-free residues and the quantities of isolated lipides are indicated in Table I.
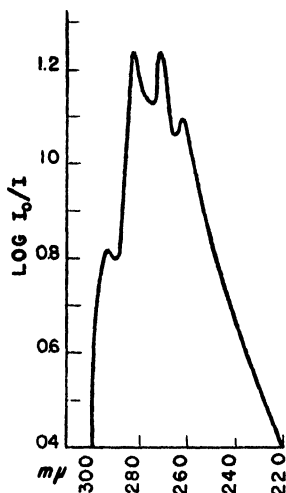
Fig. 1. Ultraviolet absorption spectrum of the unsaponifiable fraction of yeast lipoprotein particles. 4.1 mg. of the unsaponifiable fraction, corresponding to 14.6 mg. of total lipides, in 50 cc. of absolute ethanol. Thickness of absorbing layer, 1 cm.

The total lipides obtained from Preparation 1 could be separated into 12 per cent of a phosphatide fraction insoluble in acetone and 84 per cent of acetone-soluble fat. Preparation 3 yielded a total lipide fraction containing N 0.52, P 1.5 per cent; the iodine value was 131, the acid number 33.4 (calculated as palmitic acid). The ultraviolet absorption spectrum of 4.1 mg. of the unsaponifiable fraction (in 50 cc. of absolute alcohol), derived from the saponification of 14.6 mg. of the total lipides, is reproduced as Fig. 1. It is in very good agreement with the absorption spectrum of ergosterol (7). When the extinction measurements of Hogness *et al.* (7) are taken into consideration, the unsaponifiable fraction contained 2.8 mg. of ergosterol, corresponding to 19 per cent of the total lipides or to 4.5 per cent of the lipoprotein preparation, No. 3.

Phosphorus Distribution

Numerous experiments, in which the defatted lipoprotein preparations were either extracted in the cold with M sodium chloride and then deproteinized with chloroform or heated at pH 5 to 100° for 60 to 120 seconds in 0.1 M sodium chloride or acetate buffer, led to the extraction of 20 to 50 per cent of the organic P present in the starting material. The major part of the phosphorylated compounds thus extracted was non-dialyzable, but no macromolecular component could be detected on ultracentrifugation.[2]

The distribution of phosphorus compounds was determined by the method of Schneider (8). The results of two estimations carried out with lipoprotein Preparation 1 are summarized in Table II. The preparations of yeast ribonucleic and of calf thymus desoxyribonucleic acids that

TABLE II

Phosphorus Distribution in Yeast Lipoprotein

Phosphorus fraction	Experiment 1	Experiment 2
	per cent of lipoprotein P	*per cent of lipoprotein P*
Acid-soluble........	12.2	16.8
Lipide...............................	26.1	24.2
Nucleic acid.....	35.1*	34.4†
"Phosphoprotein"..	12.2	12.8
Total recovery......	85.6	88.2

* 15 per cent of the phosphorus of this fraction was found to be desoxyribonucleic acid P.

† The nucleic acid distribution in this fraction was ribonucleic acid P 86, desoxyribonucleic acid P 14 per cent.

served as standards for the colorimetric determinations have been described previously (9, 10).

DISCUSSION

Submicroscopic particles of yeast cells, isolated by high speed centrifugation of extracts of the crushed cellular material, were first described by Brachet and Jeener (11) and by Chantrenne (12). This work placed the emphasis on the presence in these particles of several enzymes (12) and of considerable quantities of firmly bound ribonucleic acid (11, 13). However, if this yeast fraction corresponds, as has been assumed (11), to the cytoplasmic particles isolated from a large number of animal tissues (14), it could be expected to be rich in lipides, which normally are found in association with this cytoplasmic fraction (3). This is actually the case, as shown in the present study.

Whether the particles from yeast are strictly analogous to those derived from the cytoplasm of animal cells is, perhaps, still open to some doubt. The crushing of animal tissue cells requires much less mechanical force than is necessary for the disintegration of microorganisms. In the latter case, there is some danger that the isolated fragments are, in their composition, representative of the whole cell rather than of a definite cellular fraction.

Although the scarcity of available material prevented a more extensive study, some of the findings on the lipide moiety of the lipoprotein particles may be of interest in this connection. The acid number agrees with the value found by Newman and Anderson (15) for yeast fat, the iodine value with the figure reported by Täufel et al. (16). The quantity of ergosterol found in the lipoprotein particles was remarkably high.

The lipoprotein particles were, in contrast to comparable fractions from animal tissues, devoid of thromboplastic activity. Yeast cephalin (17) has previously been found to have some thromboplastic effect (18).

The experiments on the detachment from the particles of ribonucleic acid, which comprises about one-third of the lipoprotein phosphorus, did not lead to the isolation of a product of a high degree of polymerization and are, therefore, not reported in detail. Attention may be directed to a study of the distribution of various phosphorylated components included in this paper.

SUMMARY

This paper discusses the isolation and composition of the submicroscopic lipoprotein particles of yeast cells. This fraction was found to contain 22 to 26 per cent of lipides, the unsaponifiable portion of which was rich in ergosterol. The composition of the lipides and the distribution of phosphorylated compounds in these particles were investigated. Almost one-third of the phosphorus was derived from ribonucleic acid, but attempts to isolate a macromolecular ribonucleic acid met with no success.

BIBLIOGRAPHY

1. Chargaff, E., and Zamenhof, S., J. Biol. Chem., 173, 327 (1948).
2. Zamenhof, S., and Chargaff, E., Science, 108, 628 (1948).
3. Chargaff, E., in Edsall, J. T., and Anson, M. L., Advances in Protein Chem., 1, 1 (1944).
4. Chargaff, E., J. Biol. Chem., 160, 351 (1945).
5. Chargaff, E., J. Biol. Chem., 161, 389 (1945).
6. Booth, V. H., and Green, D. E., Biochem. J., 32, 855 (1938).
7. Hogness, T. R., Sidwell, A. E., Jr., and Zscheile, F. P., Jr., J. Biol. Chem., 120, 239 (1937).
8. Schneider, W. C., J. Biol. Chem., 161, 293 (1945).

9. Vischer, E., and Chargaff, E., *J. Biol. Chem.*, **176, 715** (1948).
10. Chargaff, E., Vischer, E., Doniger, R., Green, C., and Misani, F., *J. Biol. Chem.*, **177, 405** (1949).
11. Brachet, J., and Jeener, R , *Enzymologia*, **11**, 196 (1943–45).
12. Chantrenne, H., *Enzymologia*, **11, 213** (1943–45).
13. Jeener, R., and Brachet, J , *Enzymologia*, **11, 222** (1943–45).
14. Hoerr, N. L., Frontiers in cytochemistry, Lancaster (1943).
15. Newman, M. S., and Anderson, R. J., *J. Biol. Chem.*, **102, 219** (1933).
16. Täufel, K., Thaler, H., and Schreyegg, H., *Z. Untersuch. Lebensmittel*, **72, 394** (1936).
17. Salisbury, L. F., and Anderson, R. J., *J. Biol. Chem.*, **112, 541** (1935–36).
18. Chargaff, E., Bancroft, F. W., and Stanley-Brown, M., *J. Biol. Chem.*, **116, 237** (1936).

A MODIFIED DIPHENYLAMINE PROCEDURE FOR THE DETERMINATION OF INULIN*

By J. MAXWELL LITTLE

(From the Department of Physiology and Pharmacology, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina)

Present methods for the determination of inulin (1–3), in which the color developed with diphenylamine is utilized, depend upon yeast fermentation for the removal of glucose from the plasma. Studies reported by Mathews (4) and some experiments of our own suggested that the destruction of glucose in an alkaline medium might be used instead of yeast fermentation in the determination of inulin. The modification reported here, in which glucose is destroyed by autoxidation in an alkaline solution, has certain advantages over other methods, *i.e.* greater simplicity and increased speed of determination.

Reagents—

Cadmium sulfate solution (5). Dissolve 34.667 gm. of $3CdSO_4 \cdot 8H_2O$ in 169.3 ml. of 1.0 N H_2SO_4 and make up to 1 liter with water.

Diphenylamine reagent (2). Dissolve 9 gm. of recrystallized diphenylamine in 300 ml. of glacial acetic acid. Add slowly 180 ml. of concentrated HCl. This reagent may be stored for a week in a dark bottle in a cool place. A slight precipitate which may form on standing does not interfere with the use of the reagent.

Sodium hydroxide solution, 1.0 N. This solution should be checked by titration.

Sodium hydroxide solution, 4 N. This solution is made from a saturated NaOH solution and should be protected from atmospheric CO_2.

Glucose solution, 100 mg. per cent. Prepare each day.

Procedure

*Plasma—*To 12 ml. of $CdSO_4$ solution in a large centrifuge tube add 1 ml. of plasma, 1 ml. of 100 mg. per cent glucose, 2 ml. of water, and 4 ml. of 1.0 N NaOH. Stopper the tube and shake vigorously for a few seconds, then occasionally for 10 minutes. Centrifuge the mixture and filter through washed cotton.

Transfer 4 ml. of the filtrate to two 200 × 25 mm. Pyrex test-tubes, and

add 1 ml. of 4 N NaOH. Cover the tubes with marbles and heat in a vigorously boiling water bath for 10 minutes. After cooling the tubes in a cold water bath for at least 2 minutes, add 10 ml. of the diphenylamine reagent to each tube; cover the tubes again and heat in a boiling water bath for 30 minutes. Cool the tubes and transfer the solution to colorimeter tubes. Determine the light transmission with an Evelyn colorimeter and a 620 mμ filter.

A blank determination on inulin-free plasma, to which 100 mg. per cent of glucose has been added, is run simultaneously with the unknown determination, and the light transmission is set at 100 per cent with the blank tube in place. For convenience, the inulin concentration may be read from a graph prepared by use of purified inulin with or without the addition of 1 ml. of 100 mg. per cent glucose.

Urine—Diluted urine (urine to plasma = 1.0) is determined in the same way as plasma. A diluted inulin-free urine blank is substituted for the plasma blank. Since it is usually necessary to dilute the urine several hundred times, variations in the urinary chromogen concentration are negligible. If the urine is free of protein and glucose, the precipitation step and the step in which the solution is heated with 1 ml. of 4 N NaOH may be omitted, in which case the urine dilution nust be adjusted appropriately.

EXPERIMENTAL

Oxygen Consumption of Glucose-NaOH Solution[1]—It will be seen in Fig. 1 that when 2 ml. of a solution of glucose and NaOH (glucose = 0.11 M, NaOH = 0.55 N) are shaken with oxygen in a Warburg vessel at 30° for 2 hours there is an appreciable oxygen consumption which is related linearly to the time of incubation. The disappearance of glucose during this time was determined chemically (6) after neutralization with H$_2$SO$_4$. At the beginning of the experimental period the flask contained 3.60 mg. of glucose, and at the end of 2 hours it contained 2.90 mg. of glucose, giving a disappearance of 0.7 mg. of glucose. The molar ratio of glucose to oxygen was 0.9.

Effect of NaOH Concentration on Glucose Destruction—4 ml. of a 1:20 dilution of a 300 mg. per cent glucose solution were heated with 1 ml. of NaOH of various concentrations. Diphenylamine was subsequently added, according to the procedure, and the determination was completed. The results, expressed as per cent light transmission, will be found in Table I. Although the transmission was usually less than 100 per cent, there was no significant increase in transmission when the NaOH concentration exceeded 0.33 N.

---

[1] Some of these experiments were done in the Department of Biochemistry, Washington University.

Effect of Time of Heating on Glucose Destruction—4 ml. of a 1:20 dilution of a 300 mg. per cent glucose solution were heated for various periods of time in a boiling water bath with 0.5 ml. of 4 N NaOH. Diphenylamine was then added, according to the usual procedure, and the determinations
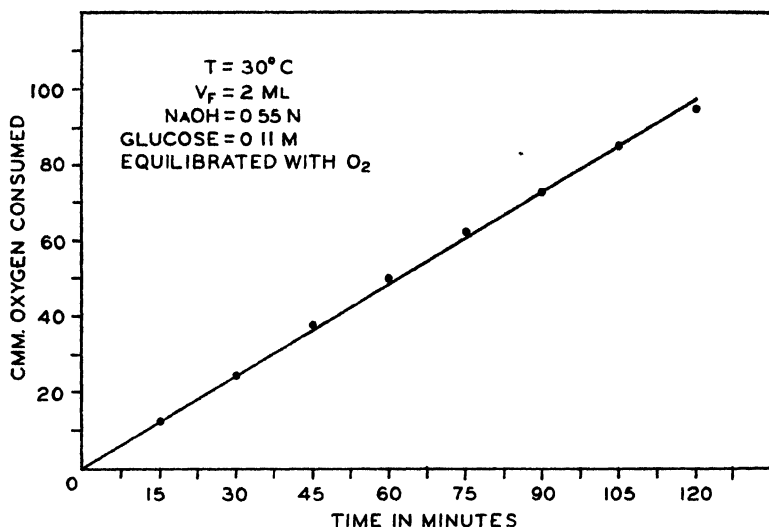
FIG. 1. Oxygen consumption of a NaOH-glucose mixture as measured in the Warburg flask. V_F = volume of fluid in the flask.

TABLE I

Effect of NaOH Concentration on Glucose Destruction

4 ml. of a 1:20 dilution of a 300 mg. per cent glucose solution plus 1 ml. of NaOH to give the indicated concentration at the time of heating.

The results are expressed as per cent light transmission with the water blank = 100 per cent.

Experiment No.	Time heated	No added NaOH	NaOH concentration at time of heating			
			0.22 N	0.33 N	0.44 N	0.60 N
	min.	*per cent*				
1	5	28.4	91.5	99.7	99.0	100.3
2	5	33.5	88.0	96.4	97.5	97.5
3	7		99.3	99.4	99.7	99.7

were completed. It will be seen in Table II that the results, expressed as per cent light transmission, are not significantly greater when the heating time exceeded 6 minutes.

Effect of Including Glucose in Blank on Apparent Glucose Destruction

with Varying Concentrations of Glucose—Filtrates were prepared with use of 1 ml. of glucose solutions of various concentrations plus 3 ml. of water. Blanks were prepared in the same manner with either water alone or 1 ml. of a 100 mg. per cent glucose solution. The determinations were carried

TABLE II

Effect of Time of Heating on Glucose Destruction

4 ml. of a 1:20 dilution of a 300 mg. per cent glucose solution plus 0.5 ml. of 4 N NaOH. NaOH concentration at the time of heating = 0.44 N.

The results are expressed as per cent light transmission with the water blank = 100 per cent.

Experiment No.	Heating time				
	3 min.	6 min	9 min.	12 min.	15 min
1	86.3		98.4	97.5	
2	82.0	99.0	99.0	99.5	
3		100.5	99.4	97.5	99.2
4		97.2	96.3	98.3	97.0
5		97.0	98.2	96.7	97.0

TABLE III

Apparent Destruction of Glucose upon Heating with NaOH When Compared with Blank Solutions Containing No Glucose and Blank Solutions Containing Equivalent of 100 Mg. Per Cent of Glucose

The heating time = 10 minutes. NaOH concentration at the time of heating = 0.8 N.

The results are expressed as per cent light transmission with the blank solutions = 100 per cent.

Experiment No.	Type of blank	Glucose concentration, mg. per cent			
		50	100	200	300
1	Water	99.4	96.3	94.0	93.0
	Glucose	101.0	101.0	98.3	97.7
2	Water	99.7	98.3	96.3	96.7
	Glucose	101.0	100.0	99.0	99.4
3	Water	97.0	97.5	97.3	94.7
	Glucose	100.0	100.5	100.5	98.0
4	Water	97.3	97.0	96.0	93.0
	Glucose	100.5	101.0	98.7	97.5

out according to the usual procedure. The colorimeter galvanometer was set at 100 per cent transmission with the water blank and the transmission of the glucose solutions was determined; then the reading was repeated with the blank representing a glucose concentration of 100 mg. per cent.

It will be seen in Table III that with a water blank the light transmission with glucose solutions was less than 100 per cent and roughly inversely proportional to the glucose concentration. With a glucose blank, however, the deviations from 100 per cent transmission were considerably less.

Effect of NaOH Concentration, at Time of Heating, on Inulin—A 20 mg. per cent inulin[2] solution was treated according to the usual procedure, except that the NaOH concentration was varied at the time of the initial heating period. The results, expressed as per cent light transmission, when compared with a water blank, will be found in Table IV. One will see that the addition of NaOH during the initial heating period resulted in a slightly higher per cent light transmission than was the case with no NaOH, but there was no significant difference in the light transmission when the NaOH concentration was varied between 0.4 N and 1.2 N.

TABLE IV

Effect of NaOH Concentration on Light Transmission of Inulin Solution

Inulin concentration = 20 mg. per cent; heating time with NaOH = 10 minutes. The results are expressed as per cent light transmission when a water blank = 100 per cent transmission.

Experiment No.	No added NaOH	NaOH concentration at time of heating		
		0.4 N	0.8 N	1.2 N
1	46.2	47.1	46.7	47.0
2	44.7	46.1	46.2	46.7
3	44.6	46.7	46.2	47.2

Recovery of Inulin from Solution Containing 100 Mg. Per Cent of Glucose—Before precipitating 1 ml. of inulin solutions of various concentrations, according to the usual procedure, 1 ml. of a 100 mg. per cent glucose solution was added to the inulin solution and to the blank. The results, read from a graph prepared from data obtained with the total procedure but with glucose-free inulin solutions, will be found in Table V. One will see that the range of recovery error was −1.3 to +4.0 per cent.

Recovery of Inulin Added to Plasma and Diluted Urine—To 1 ml. of inulin solutions of various concentrations were added 1 ml. of 100 mg. per cent glucose and 1 ml. of plasma, or urine which had been diluted 1:500. After the further addition of 1 ml. of water, the solution was precipitated with $CdSO_4$-NaOH. Then the usual procedure was followed. Plasma and urine blanks were prepared in the same manner with 1 ml. of water replacing the inulin solution. The inulin concentrations were read from

[2] The author is indebted to Dr. Marjorie Swanson of the Biochemistry Department for the purified preparation of inulin.

the graph used above. In Table VI one will see that the greatest variation of the mean recovery from the theoretical value was 2 per cent and

<div align="center">TABLE V</div>

<div align="center">Recovery of Inulin Added to Glucose Solution</div>

The blank solution and the inulin solutions contain the equivalent of 100 mg. per cent of glucose. The inulin concentrations were read from a graph prepared from the results obtained by use of standard inulin solutions without added glucose. The heating time was 10 minutes in 0.8 N NaOH.

Experiment No	Inulin added	Inulin recovered	Recovery error
	mg. per cent	*mg per cent*	*per cent*
1	5	5.0	0
	10	10.4	+4.0
	15	14.8	−1.3
	20	20.4	+2.0
	30	30.0	0
2	5	5.05	+1.0
	10	10.4	+4.0
	15	15.2	+1.3
	20	20.3	+1.5
	30	31.0	+3.3

<div align="center">TABLE VI</div>

<div align="center">Recovery of Inulin Added to Plasma and Diluted Urine</div>

	Theoretical inulin concentration	No. of determinations	Recovery range	Mean recovery, $\bar{x}$	Standard deviation of mean recovery, σ	Coefficient of variation, $\dfrac{\sigma \times 100}{\bar{x}}$
	mg per cent		*mg. per cent*	*mg. per cent*		
Plasma	5	9	4.7– 5.5	5.10	0.293	5.75
	10	9	9.5–10.6	9.93	0.357	3.60
	15	9	14.1–16.2	15.21	0.664	4.37
	20	9	18.6–21.6	20.25	0.846	4.18
	30	9	29.5–31.5	30.53	0.700	2.29
Urine diluted 1:500	5	9	4.6– 5.3	4.98	0.235	4.72
	10	9	9.2–10.0	9.78	0.272	2.78
	15	9	14.6–15.2	14.94	0.224	1.50
	20	7	19.8–20.9	20.16	0.479	2.38
	30	9	29.9–31.3	30.49	0.523	1.72

that the range of the coefficient of variation for plasma was 2.29 to 5.95; the range of the coefficient of variation for diluted urine was 1.50 to 4.72.

<div align="center">DISCUSSION</div>

When the light transmission of glucose solutions, which had been heated for 10 minutes in an alkaline medium, was compared with the trans-

mission of a water blank, it was found that there was a slight residual absorption in the glucose solution, and that this residual absorption was roughly related to the original glucose concentration. It is not known whether this residual absorption is due to a small glucose fraction which escaped destruction or whether it is due to the formation of some intermediate compound with slight absorption at this wave-length. It was shown, in Table II, that this residual absorption was not affected by increasing the time of heating. It was possible, however, to make an empirical correction for residual absorption, especially through a range of glucose concentration of from 50 to 200 mg. per cent, by adding glucose to the blank (Table III). For this reason, 1 ml. of a 100 mg. per cent glucose solution has been added to all plasma and urine samples before precipitation to insure a minimum equivalent concentration of 100 mg. per cent of glucose.

If one were certain that the glucose concentration would not change appreciably *in vivo* or *in vitro* during a determination of inulin clearance, as would be the case usually, then the addition of glucose to the samples and blank would not be necessary, for the blank would compensate for the glucose present in the samples. However, if the glucose concentration did change appreciably, then some error might be introduced. In Table III, the average inulin equivalent of the 200 mg. per cent glucose solution is 1.05 mg. when compared with a water blank, while it is 0.23 mg. of inulin when compared with a blank containing the equivalent of 100 mg. per cent of glucose.

SUMMARY

The diphenylamine method for the determination of inulin in plasma and urine has been modified by substituting autoxidation of glucose in an alkaline solution for the usual destruction of glucose by fermentation.

When glucose solutions were treated with NaOH and heated in a boiling water bath there was a slight residual light absorption which was roughly related to the glucose concentration, but which was not affected by the duration of heating. This residual light absorption was minimized by adding glucose, equivalent to 100 mg. per cent, to the blank which was used to set the colorimeter galvanometer at 100 per cent transmission. For this reason, 1 ml. of 100 mg. per cent glucose has been added to all plasma and urine samples at the time of protein precipitation.

BIBLIOGRAPHY

1. Alving, A. S., Rubin, J., and Miller, B. F., *J. Biol. Chem.*, **127**, 609 (1939).
2. Harrison, H. E., *Proc. Soc. Exp. Biol. and Med.*, **49**, 111 (1942).
3. Corcoran, A. C., and Page, I. H., *J. Biol. Chem.*, **127**, 601 (1939).
4. Mathews, A. P., *J. Biol. Chem.*, **6**, 3 (1909).
5. Fujita, A., and Irvatake, D., *Biochem. Z.*, **242**, 43 (1931).
6. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100**, 695 (1933)

THE FLUOROMETRIC MEASUREMENT OF THE NUCLEOTIDES OF RIBOFLAVIN AND THEIR CONCENTRATION IN TISSUES

By OTTO A. BESSEY, OLIVER H. LOWRY, AND RUTH H. LOVE

(From The Public Health Research Institute of The City of New York, Inc., New York, the Department of Biological Chemistry, College of Medicine, University of Illinois, Chicago, and the Department of Pharmacology, Washington University School of Medicine, St. Louis)

Warburg and Christian (1), in describing the preparation of flavin-adenine dinucleotide (FAD), mentioned that whenever dinucleotide solutions developed a greenish fluorescence they were found to have lost their coenzyme activity. On further investigation of this phenomenon it has been found that there occurs a 10-fold increase in fluorescence when FAD is split to riboflavin phosphate (flavin mononucleotide) or to free riboflavin. This change in fluorescence has proved to be a practical means of measuring FAD. Since flavin mononucleotide (FMN) can be distinguished from riboflavin on the basis of its distribution coefficient between benzyl alcohol and aqueous solutions (2), ready analytical means are available for measuring separately FAD, FMN, and riboflavin, the three forms of riboflavin encountered in biological material.

A description is given below for the fluorometric measurement of these three forms of riboflavin. The measurements are considered to be much simpler than the enzymatic procedures which were the available methods hitherto. Data are given for the fluorescent behavior of FAD, FMN, and riboflavin, the stability of FAD, and the concentration of each of these three forms of riboflavin in normal and riboflavin-deficient tissues of the rat.

Measurement of Riboflavin, FMN, and FAD in Tissues

Extraction of Flavin Compounds—The fresh tissue sample is ground in a mortar or blended (in a Waring blendor) with 25 to 50 times its volume of ice water. The cold suspension, or an aliquot, is mixed immediately with an equal volume of ice-cold 20 per cent trichloroacetic acid (final concentration 10 per cent). After 15 minutes, the sample is centrifuged and an aliquot of the extract is neutralized at once with one-fourth its volume of 4 M K_2HPO_4. Until neutralized, the sample is kept as cold as possible to prevent hydrolysis of FAD (Fig. 1). A second aliquot of the suspension is stored in the dark at 38° overnight, or at room temperature for 2 days, to produce complete hydrolysis of FAD to FMN. After

hydrolysis, this sample is neutralized in exactly the same manner as the initial sample. Considerable care is taken with both samples after neutralization to prevent undue exposure to light, since both riboflavin and FMN are much more sensitive to destruction by light in the presence of this high salt concentration than they are in dilute salt solutions. The high tissue dilution (50- to 100-fold) is necessary to effect complete flavin extraction. A lower dilution (10-fold) results in only 80 to 90 per cent recovery of the flavins.
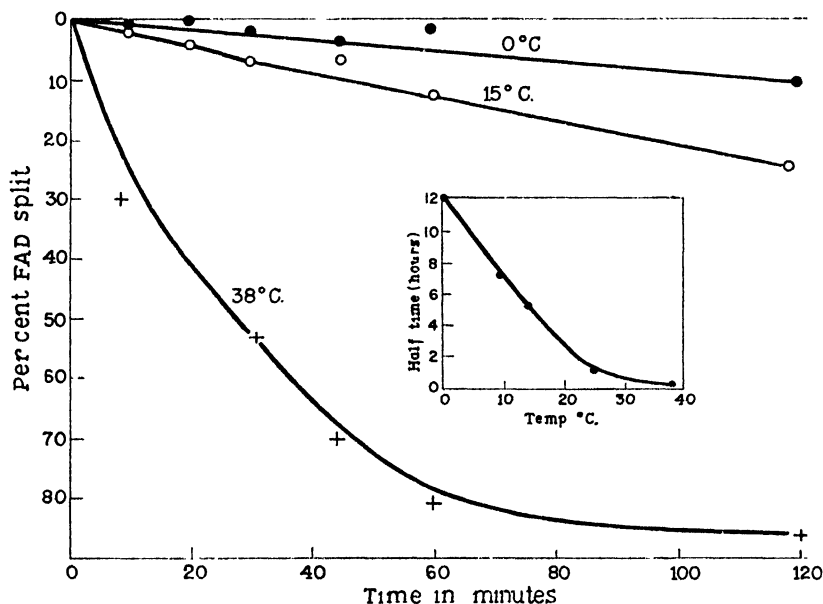
Fig. 1. Rate of splitting of FAD by 10 per cent trichloroacetic acid at different temperatures.

Measurement of FAD—The fluorescence of the two neutralized extracts is determined in the usual manner (3). An aliquot of suitable volume is measured into a fluorometer cuvette and readings are made: (*a*) initially $= F_1$; (*b*), after the addition of a standard amount of riboflavin approximately equal to that present, $= F_2$; and (*c*), after reduction with 1 per cent of the volume of 10 per cent sodium hydrosulfite in 5 per cent $NaHCO_3$, $= F_3$.

The second and third readings are corrected for the dilution of the sample with standard and reducing agents to give F_2' and F_3'. The apparent riboflavin in the aliquot is calculated as follows: Apparent riboflavin = standard riboflavin $\times (F_1 - F_3')/(F_2' - F_1)$.

In aqueous solutions, FAD is only 9 per cent as fluorescent as riboflavin

(see below). However, under the conditions of these measurements (high salt concentration) FAD (calculated as riboflavin) has a fluorescence equal to 15 per cent of riboflavin, whereas FMN (calculated as riboflavin) and riboflavin are equal in fluorescence. Therefore, if the apparent riboflavin of the initial sample is R_i and the apparent riboflavin of the hydrolyzed sample is R_t, FAD $= (R_t - R_i)/0.85$. The balance of the flavin consists of FMN plus free riboflavin. Total riboflavin $(R_t) =$ FAD $+$ non-FAD riboflavin (FMN $+$ free riboflavin).

Measurement of Free Riboflavin—Since, as is shown below, there is ordinarily very little free riboflavin in tissues, *i.e.* non-FAD flavin $=$ FMN, the above measurements will suffice for most purposes. If it is, however, desirable to distinguish between FMN and riboflavin, the procedure is as follows: An aliquot of the initial (non-hydrolyzed) neutralized sample

TABLE I

Partition Coefficients for Riboflavin and Derivatives between Benzyl Alcohol and Various Aqueous Solutions

Ratios of benzyl alcohol to aqueous layer (25–28°).

Aqueous layer	Riboflavin	FMN	FAD
0.008 M phosphate buffer, pH 6.8	3.3	0.010	0.004
4 volumes 10% CCl_3COOH + 1 volume 4 M K_2HPO_4 (final pH = 6.6)	4.1	0.032	0.020
1.5 M $(NH_4)_2SO_4$ in 2.5% CCl_3COOH and 0.45 M K_2HPO_4 (final pH 6.6)	24.0	0.082	0.111

is thoroughly shaken with an equal volume of water-saturated, redistilled, c.p. benzyl alcohol, in a glass-stoppered centrifuge tube. An aliquot of the benzyl alcohol layer is diluted in a fluorometer cuvette with 2 or more volumes of 45 per cent ethyl alcohol, which is 0.05 M in both sodium acetate and acetic acid. The apparent riboflavin in this solution is measured in this sample exactly as before (R_{Bz}). The partition coefficients for riboflavin, FMN, and FAD between benzyl alcohol and 10 per cent trichloroacetic acid, neutralized as described, are 4.1, 0.032, and 0.020 respectively (Table I). Since FAD has a fluorescence approximately 60 per cent as large as free riboflavin, when present in a mixture of benzyl alcohol and 45 per cent alcohol, the apparent riboflavin in the benzyl alcohol extract $(R_{Bz}) = 4.1/5.1$ free riboflavin $+ 0.032/1.032$ FMN $+ 0.02/1.02 \times 0.6$ FAD. By letting R non-FAD $=$ FMN $+$ free riboflavin, and solving, free riboflavin $= 1.30 R_{Bz} - 0.040 R$ non-FAD $- 0.015$ FAD.

The concentration of free riboflavin is so low in normal tissues that a special fluorometer is required to measure the fluorescence (4). In addition, it is desirable to avoid the fluorescence of even redistilled benzyl

alcohol by driving the extracted riboflavin back into an aqueous solution. This is accomplished by shaking 1 volume of the benzyl alcohol extract in a glass-stoppered vessel with 15 volumes of toluene and 1 volume of water which is 0.05 M in both sodium acetate and acetic acid. All of the flavins extracted by the benzyl alcohol are quantitatively driven into the aqueous layer; hence, the calculation is made as described above, except that the FAD correction term is changed from -0.015 to -0.003 because of the lesser fluorescence of FAD in water. The benzyl alcohol and toluene are prepared before use by shaking with large volumes of water to remove possible traces of water-soluble fluorescent materials.

The above analytical procedures would appear to be applicable, with appropriate modifications, to materials other than animal tissues.[1]

Properties of Flavin Nucleotides

FAD— The fluorescence of the FAD, used as originally prepared,[2] increased about 6-fold on hydrolysis. It was thought possible that even the small initial fluorescence might be due to contamination with free riboflavin or FMN. A sample of FAD was accordingly submitted to a counter-current extraction process (6) (with individual glass tubes) between 2 M ammonium sulfate in 0.02 N NH$_4$OH and water-saturated benzyl alcohol. After a total of eighteen plates, the maximum FAD was found in the fourth tube. The initial fluorescence values, expressed as the per cent of the fluorescence after hydrolysis in the third, fourth, and fifth tubes, were 9.2, 9.2, and 8.9 respectively. It, therefore, seems strongly indicated that pure FAD in water at a neutral pH is about 9 per cent as fluorescent as free riboflavin. It is, of course, barely possible that part of this fluorescence is still due to the presence of an impurity.

It is rather unexpected that FAD should be less fluorescent than riboflavin or FMN, since it seems likely that the FMN and adenylic acid moieties are joined through their respective phosphate groups, i.e. at a

---

[1] Other means of hydrolyzing FAD may be more expedient in certain cases. Complete hydrolysis may be effected by heating for 10 minutes at 100° in 5 or 10 per cent trichloroacetic acid or in 0.1 N HCl. The light must of course be kept very dim while the sample is hot to avoid destruction of riboflavin. An alternative method of hydrolysis is to add FAD-splitting enzyme from potato to the neutralized sample (Lowry, O. H., Bessey, O. A., and Love, R. H., in preparation, and Kornberg (5)). In this case the concentration of trichloroacetate should be kept as low as possible to minimize inhibition of the enzyme. With low substrate concentration the velocity of reaction increases with increasing acidity to a pH of about 4. An advantage of the enzymatic splitting is that a single sample may be used to measure both FAD and total riboflavin.

[2] The FAD used was prepared from yeast by the method of Warburg and Christian (1) to a purity of about 60 per cent, judging from its riboflavin content. The absorption spectrum (peaks at 452, 375, and 265 mμ) was in agreement with this purity.

point quite remote from the fluorescent isoalloxazine nucleus. If the phosphate groups are the point of union, the limited fluorescence of FAD would indicate a second linkage between the isoalloxazine nucleus and some group of the adenylic acid.

Evidence that a loose linkage, possibly electrovalent, may exist is furnished by the behavior of FAD at different pH values and in different solvents. As the pH of an aqueous solution of FAD is decreased, the fluorescence increases reversibly to a maximum at pH 2.9 and then decreases in parallel with riboflavin itself. The fluorescence *relative* to
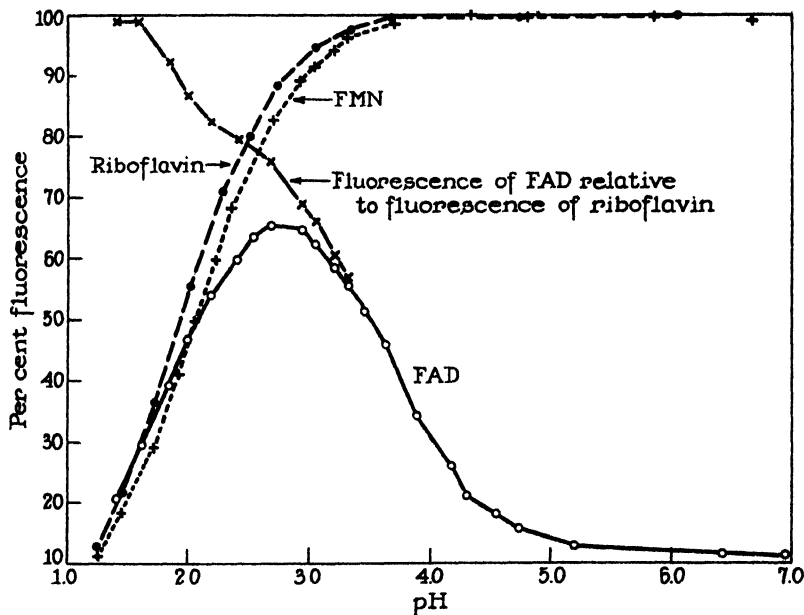
Fig. 2. Influence of pH on fluorescence of riboflavin, flavin mononucleotide, and flavin-adenine dinucleotide (FAD).

riboflavin increases steadily until the two compounds are equally fluorescent at about pH 1.5 (Fig. 2). This behavior would be compatible with a salt linkage between a positively charged group on one-half of the molecule and a negatively charged group with a pK_a of about 3.5 on the other part. The data fit this interpretation quantitatively only in the less acid part of the pH range (Fig. 2). The behavior of FAD in organic solvents is consistent with the above interpretation (Table II). Various combinations of alcohol and benzyl alcohol increase the fluorescence of FAD in the neutral range to more than 50 per cent of that of riboflavin. It is well known that lowering the dielectric constant of the medium decreases

the dissociation constant of those acids which become negatively charged on ionization (7). (The pH effect on the fluorescence, of course, does not prove the presence of an electrovalent bond, since secondary bonding of other sorts might be similarly affected.[3])

Weil-Malherbe (8) has reported that various purines are able to quench the fluorescence of a number of compounds, including riboflavin. Accordingly, the fluorescence of riboflavin and its derivatives was measured in the presence of adenosine and yeast or muscle adenylic acid. Quenching was, in fact, observed (Table III). Adenine (not shown) had a similar effect. The quenching could be reversed by lowering the pH, and this pH effect was quantitatively equal to that found for FAD (Fig. 2). It seems likely, therefore, that adenylic acid is responsible for the inhibition

TABLE II

Fluorescence of Flavin-Adenine Dinucleotide in Various Solvents

The fluorescence is expressed as per cent of fluorescence of free riboflavin in the same solvent. Ph, 0.01 M phosphate buffer, pH 6.8; Et, ethyl alcohol; Ac, 0.1 M acetate buffer, pH 4.6; and Bz, benzyl alcohol.

Solvent	Fluorescence	Solvent	Fluorescence
4 M $(NH_4)_2SO_4$	6		
100% Ph	9	55% Ac, 45% Et	40
85% " 15% Et	22	43% " 45% " 12% Bz	66
75% " 25% "	31	33% " 45% " 22% "	70
55% " 45% "	42	43% Ph, 45% " 12% "	52
5% " 95% "	48	33% " 45% " 22% "	64

of fluorescence in FAD. Although the absolute concentration of adenylic acid is far too low in a dilute FAD solution to explain the quenching, the local "concentration" of adenylic acid in the neighborhood of the riboflavin portion of the FAD molecule would quite probably be high enough to produce the phenomenon. It may be of interest to note that the presence of a high muscle adenylic acid concentration did not permit FMN to substitute for FAD as a coenzyme for D-amino acid oxidase.

The reversible increase in fluorescence of FAD at pH 2 to 3 could be put to analytical use, but it has been found more convenient, in general, to hydrolyze FAD before measurement, as described above.

Fig. 1 records the rate of hydrolysis of FAD at different temperatures

[3] Dr. C. W. Sondern of the White Laboratories, Inc., Newark, New Jersey, kindly furnished us with a synthetic compound which is believed to be diriboflavin diphosphate with a pyrophosphate linkage, as in FAD. This compound showed a 50 per cent increase in fluorescence after mild hydrolysis, which suggests a similar secondary linkage.

in 10 per cent trichloroacetic acid (pH 0.67). These data are useful for the preparation of tissue extracts by serving as a guide to permissible times of exposure of the samples to acid, if significant splitting of FAD is to be avoided. They also indicate the necessary time required at higher temperatures for complete hydrolysis. At 90°, in 0.075 N HCl, FAD is 50 per cent hydrolyzed in 2.2 minutes. Abraham (9) measured the liberation of adenylic acid under nearly comparable conditions and observed a similar rate of hydrolysis. FAD is hydrolyzed approximately 10 times faster than diphosphopyridine nucleotide under the same conditions (10). The instability of FAD is further indicated by the fact that adsorption on Florisil at a neutral pH followed by elution with organic solvents results in nearly complete conversion to FMN.

TABLE III

Quenching of Fluorescence of Riboflavin and Its Derivatives by Adenosine and Adenylic Acid

The values are reported as per cent of fluorescence in 0.008 M phosphate buffer, pH 6.8.

Medium	Riboflavin	FMN	FAD
Adenylic acid,* 0.03 M, pH 6.8.	38	49	66
" " 0.15 " " 6.8...	20	14	36
Adenosine, 0.04 " " 6.8...	28	28	45
" 0.08 " " 6.8.........	12†	17	31

* Yeast adenylic acid; muscle adenylic acid also quenched riboflavin fluorescence.
† This rose to 25 per cent at pH 3.5 and 44 per cent at pH 2.9.

The rate of increase of fluorescence of a trichloroacetic acid extract of rat liver held at 38° was found to be the same as for a similar solution of purified FAD from yeast (Fig. 3). This is strong evidence for the validity of the proposed method for FAD. Since both the liver extract and purified FAD increased in fluorescence according to a single monomolecular curve (Fig. 3), it would appear that a single molecular species is concerned in both instances.

One further property of FAD which is of some interest is its relative insensitivity to light. Under comparable conditions of exposure to ultraviolet light of wave-length 365 mμ, riboflavin and FMN are destroyed 20 times faster than FAD.

FMN—On mild hydrolysis of FAD, the fluorescence increases to a maximum with the liberation of little or no free riboflavin, as judged by the failure of benzyl alcohol to extract more than small amounts of fluorescent material. The fluorescent compound liberated is presumably FMN. Abraham (9) has shown that the product of mild acid hydrolysis is active as a

coenzyme for the "old yellow enzyme" and is, therefore, probably FMN. It will also be shown below that the hydrolytic product has the same partition coefficient as the "FMN" of tissues. More drastic hydrolysis (autoclaving in 0.1 N HCl for 15 minutes at 15 pounds pressure) induces little further change. Kuhn and Rudy (11) also noted that FMN is but slowly hydrolyzed by acid. Prolonged autoclaving with higher acid concentration results in only partial hydrolysis of FMN with accompanying destruction of part of the riboflavin. Treatment with acid phosphatase (clarase) liberates free riboflavin with no change in fluorescence. Thus, FMN has
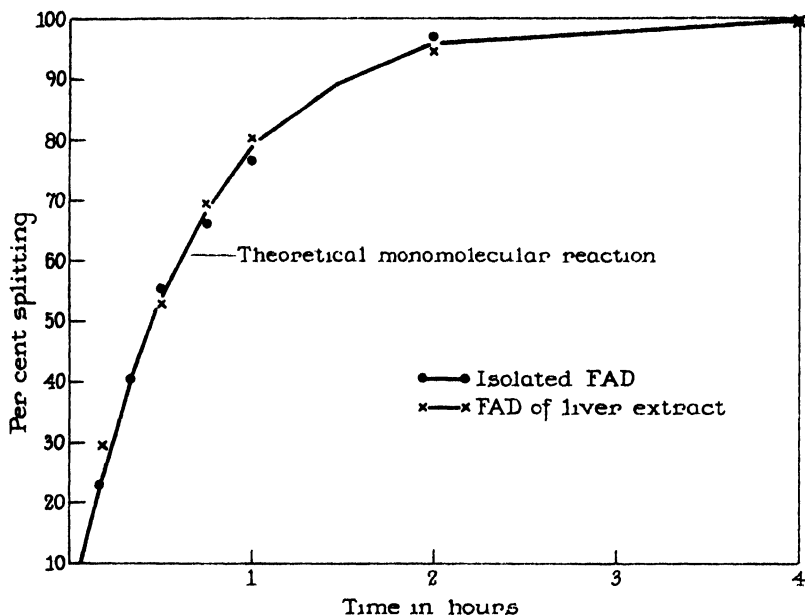
FIG. 3. Splitting of isolated FAD and FAD present in an extract of rat liver by 10 per cent trichloroacetic acid at 38°.

the same fluorescence as riboflavin. There is, however, a slight difference in the effect of acid on the fluorescence of FMN and of free riboflavin (Fig. 2). The phosphorylated compound loses its fluorescence at a slightly higher pH than does riboflavin. Thus, at pH 2 riboflavin is 18 per cent more fluorescent than FMN. The loss of fluorescence of both compounds fits a titration curve almost perfectly. Assuming, therefore, that this change represents the conversion of a single group to an acid, this group has a pK_a of 1.97 in riboflavin and a pK_a of 2.17 in FMN. (Kuhn and Moruzzi (12) estimated a pK_a of about 1.7 for riboflavin from the effect of pH on its fluorescence.)

Partition Coefficients of Flavins—Emmerie (2) used the difference between the benzyl alcohol-water partition coefficients of riboflavin and FMN to separate these two compounds. The differences in the distribution of the flavin compounds between aqueous solutions and benzyl alcohol are useful for both analytical and characterization purposes (Table I). FMN is more readily extracted than FAD from dilute salt solutions, whereas the reverse is true from strong ammonium sulfate solutions.
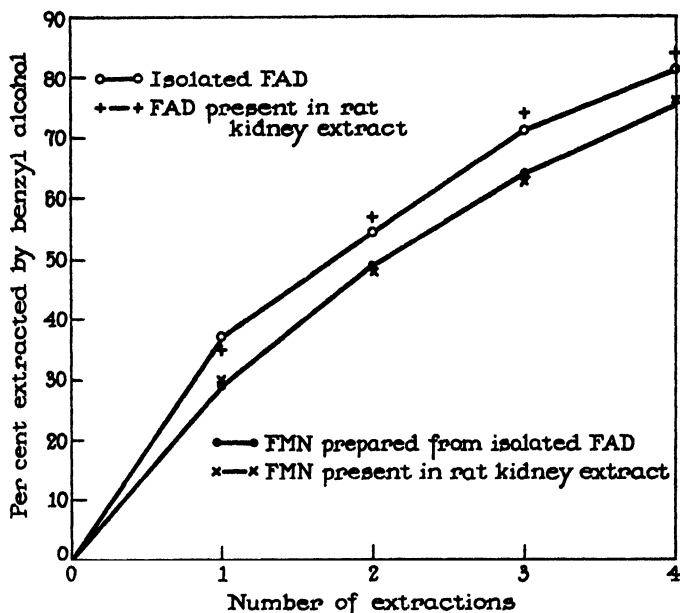
FIG. 4. Extraction of FMN and FAD by benzyl alcohol from extracts of rat kidney. A 10 per cent trichloroacetic acid extract of rat kidney was neutralized with K_2HPO_4, made 1.5 M in $(NH_4)_2SO_4$, and extracted four times with 5 volumes of benzyl alcohol.

A neutralized trichloroacetic acid extract of rat kidney was brought to a concentration of 1.5 M in ammonium sulfate and extracted successively with 5-fold volumes of benzyl alcohol, and the FMN and FAD left behind were measured by the proposed method. The extraction curves (Fig. 4) concur within experimental error with similar curves found for purified yeast FAD and for FMN obtained by mild hydrolysis from this yeast FAD. These data indicate the identity of the rat kidney flavins with the purified compounds and suggest that no significant amounts of riboflavin derivatives other than these two exist in rat kidney. These data may also be regarded as further proof for the validity of the proposed analytical procedure.

Stability of FAD in Tissues and Tissue Extracts—In measuring the FAD in tissues there are two possibilities of loss other than incomplete extraction, *viz.* enzymatic splitting of FAD prior to extraction and acid splitting during extraction. There exists an enzyme or enzymes in at least kidney and liver capable of splitting FAD (13). This enzymatic effect is apparently less marked in the intact tissue than in tissue mince (Table IV). The data indicate that even kidney mince, which is quite active in this respect, may safely be allowed to stand for 30 minutes at 10°. There is a greater danger of hydrolysis after the addition of 10 per cent trichloroacetic acid (Table IV; Fig. 1). To prevent serious loss it appears that the acid extracts should be neutralized within 60 minutes at 0°, 30 minutes at 10°, or 15 minutes at 15°.

Comparison of Coenzymatic and Fluorometric Assay for FAD—FAD was measured in a number of tissues by its function as a coenzyme for D-amino

TABLE IV

Stability of FAD in Rat Kidney and Kidney Extracts

	Tempera-ture	FAD remaining		
	°C.	*15 min. per cent of initial*	*30 min. per cent of initial*	*60 min. per cent of initial*
Intact kidney.....	38		99	
Kidney blended with water.	10	100	102	95
" " " "	38	66	59	40
" 1:50 extract in 10% CCl₃COOH....	15	93	87	84

acid oxidase. The apoenzyme was prepared from pig kidneys by essentially the method described by Warburg and Christian (1).

The assay was conducted in 0.05 N pyrophosphate buffer at pH 8.3, with 3 mg. of DL-alanine per ml. as substrate and fluorometrically assayed yeast FAD of 60 per cent purity as a standard. The concentration of FAD required for half activity was found to be 0.155 γ per ml. as compared to 0.196 γ per ml. reported by Warburg and Christian (1) for enzyme from sheep kidney. The tissues were blended in ice water and then heated at 100° for 5 minutes to liberate the FAD. Aliquots of the supernatant fluids after centrifuging were assayed for their FAD content by the enzymatic procedure and the values found were compared with the values obtained fluorometrically on the same extracts (Table V). The data obtained by the two procedures are seen to concur within experimental limits. Similar comparative analyses were made on a sample of FAD which was heated for different lengths of time in 0.075 N HCl at 90°. The decrease in FAD as measured fluorometrically was found to be accompanied by a correspond-

ing fall in coenzymatic activity, thus further demonstrating the relationship between potential fluorescence and FAD activity.

<div align="center">TABLE V</div>

Comparison of Values for FAD Obtained by Fluorometric and Enzymatic Methods
The values are based on wet tissue weight.

	Fluorometric		As coenzyme*
	Free riboflavin plus FMN	FAD (as riboflavin)	FAD (as riboflavin)
	γ per gm.	γ per gm.	γ per gm.
Liver 1	7.0	28.3	28.5
" 2	5.7	36.8	34.9
Kidney 1	21.8	19.2	21.8
" 2	17.0	16.4	13.8
Heart 1	1.5	18.1	17.6
" 2	2.4	21.6	18.8
Average		23.4	22.6

* For D-amino acid oxidase.

<div align="center">TABLE VI</div>

Recovery of Flavins Added to Minced Liver

Substance	Initial	Addition	Found	Calculated	Recovery	Substance	Initial	Addition	Found	Calculated	Recovery
	γ per gm.	γ per gm.	γ per gm.	γ per gm.	per cent		γ per gm.	γ per gm.	γ per gm.	γ per gm.	per cent
Riboflavin	0.6	10.3	10.9	10.9	100	FAD	31.6	38.8	67.8	70.4	93
"	0.6	20.3	20.0	20.6	97	"	31.6	38.8	66.2	70.4	91
						"	31.6	38.8	72.0	70.4	104
Average					98	"	33.3	29.6	59.7	62.9	89
						"	33.3	29.6	55.0	62.9	73*
FMN	6.0	14.7	18.1	20.7	82	"	29.9	39.0	69.2	68.9	101
"	6.0	14.7	19.3	20.7	91	"	29.9	39.0	64.5	68.9	89
"	7.9	15.0	25.1	22.9	115						
"	7.9	15.0	25.4	22.9	117	Average					95
"	13.5	31.4	43.9	44.9	97						
						Riboflavin	0.6	9.9	12.6	10.5	121
Average					100	FMN	4.9	14.6	20.7	19.5	108
						FAD	29.6	73.5	109.0	103.1	108
						Total	35.1	98.0	142.3	133.1	108

* Omitted from the average.

Recovery of Flavins Added to Tissues—Riboflavin, FMN, and FAD were added to the cold minced tissues before extraction with trichloroacetic acid. The recovery of the separate flavins averaged 95 to 100 per cent (Table

VI). Recovery was, however, less complete (85 per cent) if the final dilution was only 1:10 instead of 1:100.

TABLE VII

Three Riboflavin Fractions of Five Tissues of Control and Riboflavin-Deficient Rats

The values are calculated as riboflavin on the basis of wet tissue weight.

	Control					Riboflavin-deficient						
	Total ribo-flavin	FAD	FMN	Free ribo-flavin	FAD	Total ribo-flavin	FAD	FMN	Free ribo-flavin	FAD	FMN	
	γ per gm.	γ per gm.	γ per gm.	γ per cent	per cent of total	γ per gm.	γ per gm.	γ per gm.	γ per gm.	per cent of total	per cent of average control	per cent of average control
Liver	39.8	32.3	6.6	0.90	81	11.8	10.9	0.7	0.24	92	36	11
	40.4	34.0	5.8	0.62	84	13.3	12.0	1.1	0.24	90	39	18
	31.4	26.6	4.8*		71	9.7	8.8	0.5	0.36	91	29	8
	33.4	24.2	9.2		72							
	40.5	35.2	5.3		87							
	37.1†	**30.5**	**6.2**	**0.76**	**79**	**11.6**	**10.6**	**0.8**	**0.28**	**91**	**35**	**12**
Kidney	38.2	23.2	14.0	1.02	61	16.2	12.6	3.3	0.28	78	53	28
	41.4	26.2	14.4	0.82	63	15.2	12.9	2.1	0.19	85	55	18
	40.5	32.4	7.2	0.92	80	18.4	16.9	1.1	0.38	92	72	9
	27.2	18.1	9.1*		66							
	26.5	18.3	8.2		69							
	34.8	**23.6**	**11.9**	**0.92**	**68**	**16.6**	**14.1**	**2.2**	**0.28**	**85**	**60**	**18**
Heart	21.6	19.5	1.9	0.19	90	7.8	6.8	1.0	0.07	86	44	50
	21.6	19.5	2.0	0.11	90	8.6	7.7	0.9	0.07	89	50	45
	21.5	17.7	3.8*		82	9.3	8.0	1.2	0.05	86	52	60
	24.4	20.6	3.8		84							
	19.3	13.8	5.5		72							
	21.7	**18.2**	**2.0**	**0.15**	**84**	**8.6**	**7.5**	**1.0**	**0.06**	**87**	**49**	**51**
Brain	2.95	2.19	0.62	0.14	74	2.04	1.56	0.40	0.08	76	63	58
	3.25	2.37	0.76	0.12	73	2.24	1.73	0.44	0.07	77	70	64
	3.47	2.62	0.74	0.11	75	1.89	1.46	0.37	0.06	77	59	54
	3.60	2.69			73							
	3.34	**2.47**	**0.71**	**0.12**	**74**	**2.06**	**1.58**	**0.40**	**0.07**	**77**	**64**	**58**
Skeletal muscle	3.56	3.13	0.43	0.00	88	0.86	0.74	0.15	-0.02	85	22	38
	3.48	3.10	0.33	0.05	89	1.24	1.08	0.09	0.07	87	32	23
	4.47	3.99	0.44	0.04	89	0.78	0.64	0.05	0.08	83	19	13
	3.84	**3.41**	**0.40**	**0.04**	**89**	**0.96**	**0.82**	**0.10**	**0.04**	**85**	**24**	**25**

* Calculated value.

† Averages.

Concentration of Individual Flavins in Normal and Deficient Tissues— The concentration of riboflavin, FMN, and FAD was measured in five major tissues of normal and riboflavin-deficient rats. The deficient rats

had been maintained for several months on purified diets containing about 5 γ of riboflavin per day. The control animals were young adults on a Purina dog chow diet. The FAD was found to represent the largest frac-

TABLE VIII

FAD and Non-FAD Riboflavin Fractions of Lung, Thymus, Submaxillary Gland, and Adrenals of Control and Riboflavin-Deficient Rats

The values are calculated as riboflavin on the basis of wet tissue weight.

	Control				Riboflavin-deficient					
	Rat No.	Total riboflavin	FAD	FMN + free riboflavin	FAD	Rat No.	Total riboflavin	FAD	FMN + free riboflavin	FAD		FMN + free riboflavin
		γ per gm.	γ per gm.	γ per gm.	per cent of total		γ per gm.	γ per gm.	γ per gm.	per cent of total	per cent of average control	per cent of average control
Lung	1	3.76	3.10	0.66	82	5	3.26	2.52	0.74	77	69	118
	2	4.22	3.66	0.56	87	6	4.24	3.76	0.48	89	103	76
	3	4.80	4.29	0.51	89	7	5.12	4.57	0.55	89	126	88
	4	4.29	3.49	0.80	81	8	3.41	3.05	0.36	89	84	57
		4.27*	3.64	0.63	85		4.01	3.48	0.53	86	96	84
Thymus	1	2.76	2.12	0.64	77	5	1.86	1.41	0.45	76	70	78
	2	2.47	2.11	0.36	85	6	1.60	1.37	0.23	86	68	40
	3	2.47	1.87	0.60	76	7	2.01	1.56	0.45	78	78	78
	4	2.67	1.95	0.72	73	8	2.41	1.68	0.73	70	84	126
		2.59	2.01	0.58	78		1.97	1.51	0.46	78	75	79
Submaxillary gland	1	6.30	5.12	1.18	81	5	4.65	3.12	1.53	67	67	137
	2	5.59	4.85	0.74	87	6	6.75	4.65	2.10	69	100	187
	3	4.94	3.67	1.27	74	7	4.98	3.93	1.05	79	85	94
	4	6.24	4.93	1.31	79	8	4.52	3.30	1.22	73	71	109
		5.77	4.65	1.12	80		5.22	3.74	1.48	72	80	132
Adrenals	1	19.9	17.6	2.3	88	5	19.3	15.1	4.2	78	83	105
	2	20.1	16.2	3.9	81	6	15.8	15.9	−0.1	101	87	
	3	25.0	20.6	4.4	82	8	17.3					
	4	24.2	18.9	5.3	78		17.5	15.4		90	85	
		22.3	18.3	4.0	82							
Liver	1	38.6	36.7	1.9	95	5	13.7	11.7	2.0	85	37	69
	2	34.5	32.7	1.6	95	6	18.9	16.8	2.1	89	53	72
	3	31.1	25.8	5.3	83	7	15.3	13.1	2.2	86	42	76
		34.7	31.8	2.9	92		16.0	13.9	2.1	87	44	72

* Averages.

tion in all of the tissues (Table VII). The percentage in the normal tissues varied from nearly 90 per cent of the total in skeletal muscle to less than 70 per cent in kidney. The balance was found to be chiefly FMN. The relatively large quantity of FMN in kidney is of interest and is quite surely

not an artifact, since preparation of extracts with the greatest haste, after killing the animal, did not decrease the percentage of FMN found. The free riboflavin was observed to be consistently low, too low in several of the tissues for accurate measurement by the technique available. The kidney had the highest absolute amount (0.7 γ per gm.) and the brain the highest relative concentration (3 per cent of the total).

In riboflavin deficiency all three fractions decreased. In liver and kidney the greatest fall was found for FMN. In heart, brain, and skeletal muscle, in contrast, both FAD and FMN decreased to about the same relative extent. It is perhaps unwise to speak too definitely about the FMN

TABLE IX

FAD and Non-FAD Riboflavin Fractions of Miscellaneous Tissues (Single Values)

The values are calculated as riboflavin on the basis of wet tissue weight.

Tissue	Control				Riboflavin-deficient rat					
	Total ribo-flavin	FAD	FMN + free ribo-flavin	FAD	Total ribo-flavin	FAD	FMN + free ribo-flavin	FAD	FAD	FMN + free ribo-flavin
	γ per gm.	γ per gm	γ per gm.	per cent of total	γ per gm.	γ per gm.	γ per gm	per cent of total	per cent of control	per cent of control
Gastric mucosa	10.9	9.3	1.6	86	5.27	4.67	0.60	89	50	38
Pancreas	8.14	6.82	1.32	84	5.67	5.28	0.39	93	77	30
Ovary	7.52	5.95	1.57	79	6.11	5.15	0.96	83	87	61
Thyroid	4.56	2.90	1.66	64	3.38	2.43	0.95	72	84	57
Spleen	3.90	3.38	0.52	87	2.63	2.35	0.28	89	69	54
Pituitary	3.68	2.62	1.06	68	2.51	1.20	1.31	48	46	128
Testes	3.37	2.45	0.92	73						
Lymph node	2.86	2.18	0.68	76	1.83	1.46	0.37	80	67	54
Uterus	2.34	1.82	0.52	78	1.99	1.81	0.18	91	99	34
Bladder	2.31	1.59	0.72	69	1.36	1.12	0.24	82	70	33
Cornea	1.12	1.00	0.12	89	0.85	0.66	0.18	78	66	150
Skin	1.00	0.76	0.24	76	0.61	0.55	0.07	89	73	68

concentration from these few data, since the values have not been found to be very consistent. Contrast, for example, the FMN data for liver found in Table VIII with those in Table VII. Unknown age or environmental factors may influence the concentration of this flavin.

Except for brain, the above five tissues are characterized by a marked drop in total riboflavin concentration in riboflavin deficiency. Not all tissues are so severely affected. Lung, thymus, submaxillary, and adrenal glands are presented as typical of tissues or organs in which deficiency produces only minor changes (Table VIII). FAD comprised about 80 per cent of the total riboflavin in these tissues and deficiency was without dramatic effect on this percentage.

Single values were obtained purely for orientation purposes on twelve other normal and deficient rat tissues (Table IX). No striking percentage of FAD or change in deficiency was noted. Until these data are obtained with more animals, they must be interpreted with caution.

SUMMARY

1. A convenient fluorometric procedure is described which permits separate measurement of riboflavin, riboflavin monophosphate, and riboflavin dinucleotide. The measurement of the dinucleotide is based on the fact that it has much less fluorescence than riboflavin, and that it can be readily converted into riboflavin monophosphate which exhibits the same fluorescence as riboflavin. Riboflavin is distinguished from its two derivatives by its very much greater partition coefficient between benzyl alcohol and water.

2. Data are presented concerning some of the properties of riboflavin dinucleotide, particularly its rate of hydrolysis in acid and the influence of pH and solvent on its fluorescence.

3. Evidence is given that riboflavin monophosphate and dinucleotide account for practically all of the riboflavin of rat kidney.

4. The riboflavin dinucleotide content of liver, kidney, and heart was measured both fluorometrically and enzymatically with concurring results.

5. The data are given for the concentration of the three riboflavin fractions in five major rat tissues, both normal and riboflavin-deficient, and for the total riboflavin and dinucleotide concentrations in sixteen others. Free riboflavin was found to be present in quantitatively insignificant amounts. The dinucleotide accounted in general for 70 to 90 per cent of the total riboflavin.

BIBLIOGRAPHY

1. Warburg, O., and Christian, W., *Biochem. Z.*, **298**, 150 (1938).
2. Emmerie, A., *Rec. trav. chim. Pays-Bas*, **58**, 290 (1939).
3. Burch, H. B., Bessey, O. A., and Lowry, O. H., *J. Biol. Chem.*, **175**, 457 (1948).
4. Lowry, O. H., *J. Biol. Chem.*, **173**, 677 (1948).
5. Kornberg, A., *J. Biol. Chem.*, **174**, 1051 (1948).
6. Craig, L. C., *J. Biol. Chem.*, **155**, 519 (1944).
7. Linderstrøm-Lang, K., *Compt.-rend. trav. Lab. Carlsberg*, **17**, No. 4 (1927).
8. Weil-Malherbe, H., *Biochem. J.*, **40**, 363 (1946).
9. Abraham, E. P., *Biochem. J.*, **33**, 543 (1939).
10. Warburg, O., and Christian, W., *Biochem. Z.*, **274**, 112 (1934).
11. Kuhn, R., and Rudy, H., *Z. physiol. Chem.*, **239**, 47 (1936).
12. Kuhn, R., and Moruzzi, G., *Ber. chem. Ges.*, **67**, 888 (1934).
13. Ochoa, S., and Rossiter, R. J., *Biochem. J.*, **33**, 2008 (1939).

MECHANISMS OF CLEAVAGE OF GLUCOSE-1-PHOSPHATE

By MILDRED COHN

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis)

In the most direct method for studying the mechanism of reactions involving the rupture of an oxygen bond, isotopic oxygen is employed as an indicator. The first such study was made by Polanyi and Szabo (1) in the alkaline hydrolysis of a carboxylic ester in water containing an excess of O^{18}. They showed that the O^{18} content of the alcohol formed in the hydrolytic reaction was normal in isotopic composition and hence did not originate from the water, thereby establishing unequivocally that cleavage occurred between oxygen and the carbon of the carboxyl group. Subsequently the mechanisms of a number of related reactions were similarly established, including acid hydrolysis of a carboxylic ester (2), the acid-catalyzed esterification reaction (3), the alkaline (4) and acid (5) hydrolysis of trimethyl phosphate, and the alkaline and acid hydrolysis of acetyl phosphate (6).

This type of approach could be of value in elucidating the detailed mechanisms of a multiplicity of reactions involving phosphate esters of biochemical interest. The present paper deals with a series of reactions of glucose-1-phosphate. It was thought of interest to compare the enzymatic and non-enzymatic hydrolyses as well as the cleavage in some non hydrolytic enzymatic reactions. The reactions investigated in this manner include the hydrolyses catalyzed by acid, by an acid phosphatase, and by an alkaline phosphatase and two reversible reactions of glucose-1-phosphate catalyzed by muscle phosphorylase and sucrose phosphorylase respectively.

Before embarking on a study of these reactions with O^{18}, it was necessary to devise a method for analyzing phosphate for its O^{18} content. It was also necessary to investigate the stability of the oxygen in phosphate with respect to exchange with water under various conditions encountered in these reactions.

EXPERIMENTAL

Preparation of $KH_2PO_4^{18}$—To 1 mM of P_2O_5, approximately 3.3 mM of H_2O^{18} (1.343 atom per cent excess O^{18}) were added. After the violent reaction had subsided, the mixture was heated gently for a few minutes and allowed to cool. Enough 2 N KOH was then added to adjust the pH

to approximately 4.4. 2 volumes of ethyl alcohol were added to precipitate KH_2PO_4. The precipitate was filtered, washed twice with ethyl alcohol, and twice with ether. The product was dried at 100° *in vacuo* for 1 hour. This method of precipitating and drying KH_2PO_4 was used in all subsequent experiments.

Determination of O^{18} Content of $KH_2PO_4{}^{18}$—2.5 mM of $KH_2PO_4{}^{18}$ prepared above were placed in a tube which was sealed into a vacuum line shown in Fig. 1. After evacuation of the system, the equilibration Vessel B was cooled with dry ice and the orthophosphate sample in Vessel A was
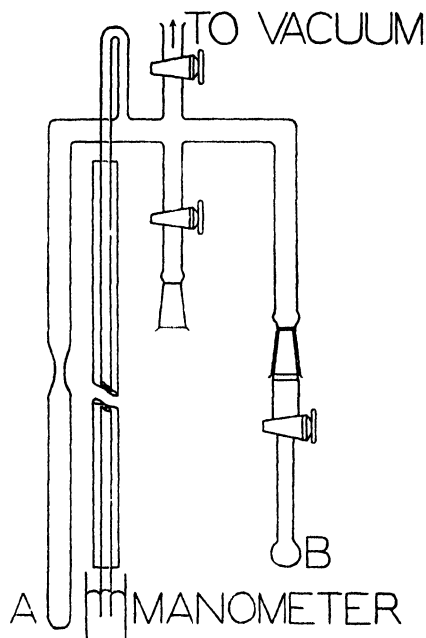
Fig. 1. Apparatus for dehydration of KH_2PO_4

converted to metaphosphate and water by heating gently with a micro burner until no more water was given off. The water was distilled into Vessel B. The isotopic content of the water was determined by equilibration with CO_2 as described by Cohn and Urey (7). The equilibrated CO_2 was analyzed for its O^{18} content with a mass spectrometer.[1]

The O^{18} content of the water obtained from the dehydration of KH_2PO_4 was found to be 0.502 atom per cent excess. The calculated value, assuming randomization of the four oxygens in phosphate, is 0.504 atom per

[1] A few of the analyses reported in this paper were made by the National Bureau of Standards and the remainder in a mass spectrometer of the Nier type (8).

cent excess, i.e., $\frac{1}{3} \times 1.343$ (the atom per cent excess of the water used to prepare the phosphate). Thus this method of preparation of labeled orthophosphate and this method of analysis of the O^{18} content proved satisfactory.

Control Experiments—The data available on exchange of phosphate with water are, at best, meager (9, 10). Table I gives the results of the

TABLE I

Control Experiments

Experiment No.	Reaction mixture	Temperature	Time	Atom per cent excess O^{18} in KH_2PO_4	
				Initial	Final
		°C.	min.		
1	3 mM KH_2PO_4 + 10 cc. 0.3 N HCl	100	10	0.502	0.466
			hrs.		
2	3 " " + 10 " 0.3 " "	100	24	0.431	0.422
3	3 " " + 10 " 2 N KOH	100	3	0.431	0.407
4	3 " " + acid phosphatase in 20 cc. solution, pH 5.2	25	24	0.431	0.446
5	KH_2PO_4, pptd. with Ba^{++} and recovered			0.358	0.389

TABLE II

Hydrolysis Reactions of Glucose-1-Phosphate

Catalyst	Atom per cent excess O^{18} in medium	Atom per cent excess in isolated phosphate		
		Found	Calculated	
			$C-O\overset{|}{\underset{|}{-}}P$	$C\overset{|}{\underset{|}{-}}O-P$
Acid .	1.222	0.00	0.306	0
	1.236	0.02	0.309	0
" phosphatase. . .	1.009	0.207	0.252	0
Alkaline phosphatase.	0.939	0.252	0.235	0

exchange reactions studied. Phosphate exchanges only slightly even under rather drastic conditions (Experiment 2). The result of Experiment 1, *i.e.* the 7 per cent loss in O^{18} concentration due to exchange of the free acid after 10 minutes at 100°, is probably too high, since subsequent experiments in which this procedure was used did not show any observable exchange and the 24 hour experiment (Experiment 2) under the same conditions indicated a loss of about 2 per cent. The procedure of Experiment 5 was that used in most experiments to isolate phosphate; the phosphate was precipitated with Ba^{++} at approximately pH 8.3,

centrifuged, and redissolved in HCl. The Ba^{++} was then removed with K_2SO_4, the pH adjusted to about 4.4 with KOH, and the KH_2PO_4 precipitated in 66 per cent ethyl alcohol. This procedure seems to cause a slight increase in isotope concentration, which was also observed in other experiments in which this procedure was employed (see Tables II and III).

Studies of Reaction Mechanism

General Procedures—Two types of experiment were carried out. In one type, exemplified by the hydrolysis reactions, the O^{18} was initially present only in the water of the medium. The inorganic phosphate obtained in the reaction was dehydrated and the O^{18} in the water obtained from dehydration of the phosphate was determined by equilibration with CO_2, as already described. In the second type of experiment, used

TABLE III

Phosphorylase Reactions

Enzyme	Atom per cent excess O^{18} in initial inorganic phosphate	Atom per cent excess O^{18} in final phosphate			
		Found		Calculated	
		Inorganic	Glucose-1-P	$C-O\overset{\mid}{\underset{\mid}{}}P$	$C\overset{\mid}{\underset{\mid}{}}O-P$
Muscle phosphorylase... ...	0.508	0.534	0.522	0	0.508
Sucrose "	0.431	0.232	0.228	0	0.216

in the study of the reversible phosphorylase reactions, the O^{18} was initially present in the inorganic phosphate. At the end of the reaction, the inorganic phosphate was separated from the glucose-1-phosphate and eventually both were obtained in the form of KH_2PO_4, which was analyzed for O^{18} in the usual manner.

Acid Hydrolysis—1 gm. of the dipotassium salt of glucose-1-phosphate was dissolved in 9.4 gm. of H_2O^{18} (approximately 1.3 atom per cent excess), to which were added 1.1 gm. of a 20.26 per cent HCl solution. The reaction mixture was sealed in a tube and heated for 12 minutes at 100°. The tube was cooled in ice and 0.2 cc. of the mixture withdrawn for equilibration with CO_2, so that the isotopic content of the medium could be determined. 1.5 cc. of 2 N KOH were then added, the solution was distilled *in vacuo* at room temperature to a small volume (approximately 2 cc.), and the phosphate precipitated as KH_2PO_4, as already described. Upon dehydration, the phosphate turned brown, from which it was concluded that the phosphate had been contaminated with glucose. The experiment was repeated and contamination was avoided in the second experiment by ascertaining that all solid matter was completely in solution

before precipitation with alcohol. As can be seen in Table II, the results of both experiments are essentially the same.

Acid Phosphatase Hydrolysis—1 gm. of the dipotassium salt of glucose-1-phosphate was dissolved in 20 cc. of a solution containing H_2O^{18}, 2 cc. of an acid phosphatase preparation from prostate, and enough hydrochloric acid so that the final pH was approximately 5.3. The course of the reaction was followed by phosphate analyses (11). After 24 hours at room temperature, the reaction had proceeded 70 per cent to completion and showed no tendency to approach completion at an appreciable rate. After 26 hours, 0.5 cc. of the medium was withdrawn for analysis for O^{18} content, the solution was distilled at room temperature *in vacuo* to about 3 cc., and the inorganic phosphate was precipitated with Ba^{++} at pH 8.3. The precipitate was dissolved in hydrochloric acid and again precipitated with Ba^{++} at pH 8.3. The barium phosphate was then converted to the monopotassium salt in the usual manner. The results of the isotopic analyses are given in Table II.

Alkaline Phosphatase Hydrolysis—1 gm. of the dipotassium salt of glucose-1-phosphate was dissolved in 9.5 cc. of 0.1 M veronal buffer in H_2O^{18}, pH 9.0, and 0.5 cc. of a purified preparation of intestinal phosphatase was added. The course of the reaction was followed by phosphate analyses. At the end of 1 hour, another 0.5 cc. of enzyme solution was added because the reaction was proceeding too slowly. After 24 hours at room temperature, the reaction was about 93 per cent complete and a sample was withdrawn for isotopic analysis of the medium. The phosphate was precipitated with Ba^{++} and converted to KH_2PO_4 in the usual manner, and its O^{18} content was determined. The analytical values for the O^{18} content of the medium and phosphate are given in Table II.

Muscle Phosphorylase Reaction—20 mM of KH_2PO_4 (0.51 atom per cent excess O^{18}) were dissolved in water; 4 gm. of glycogen and 0.313 gm. of cysteine hydrochloride were added. The solution was adjusted to pH 7.3 with KOH and 2 cc. of a crystalline muscle phosphorylase *a* suspension (12) were added. The total volume was 25 cc. Equilibrium between the glucose-1-phosphate formed and the inorganic phosphate was attained in 2 hours, as determined by phosphate analyses. The reaction was allowed to proceed for 4 hours to insure complete equilibration of the oxygen. The two phosphate compounds were separated by a procedure previously described (13). The inorganic phosphate was precipitated twice as the barium salt and finally as the monopotassium salt. The glucose-1-phosphate fraction contained a small amount of inorganic phosphate but no attempt was made to remove the contamination, since both fractions should have the same O^{18} concentration at equilibrium. It was possible to determine the O^{18} content of glucose-1-phosphate by hy-

drolyzing it in acid and analyzing the inorganic phosphate formed, since it had already been shown that, in the acid hydrolysis of glucose-1-phosphate, the oxygen is retained in the inorganic phosphate (see Table II). The glucose-1-phosphate was hydrolyzed for 10 minutes at 100° in 1 N acid to obtain its phosphate finally in the form of KH_2PO_4. The O^{18} concentration of the initial and final inorganic phosphate and of the phosphate of the glucose-1-phosphate formed is listed in Table III.

Sucrose Phosphorylase Reaction--It has been shown by Doudoroff, Barker, and Hassid (14) that the enzyme sucrose phosphorylase effects an exchange of phosphate between glucose-1-phosphate and inorganic phosphate in the absence of an acceptor. The first step in any reaction catalyzed by this enzyme is the reversible cleavage of the phosphate bond. The simplest reaction in which to study this cleavage is therefore the exchange of phosphate between glucose-1-phosphate and inorganic phosphate. However, since there is no net change in amount in the components of the reaction system, it was necessary to use radioactive P as an indicator for the extent of the reaction.

4 cc. of a dialyzed sucrose phosphorylase solution, prepared from 1.5 gm. of dried bacteria essentially by the method of Doudoroff (15), were added to approximately 3 mM of the dipotassium salt of glucose-1-phosphate containing radioactive P and 3 mM of monopotassium phosphate containing O^{18}; the total volume was adjusted to 30 cc. After 17 hours at room temperature, the inorganic and ester phosphates were separated as in the preceding experiment with muscle phosphorylase. The radioactivity of each fraction was determined and the glucose-1-phosphate was found to have 6350 counts per minute per mg. of P and the inorganic phosphate 5950 counts per minute per mg. of P. These values indicated that the reaction had approached close to equilibrium. The O^{18} analyses of the two phosphates as shown in Table III similarly indicate almost complete equilibration.

DISCUSSION

The mechanism of the rupture of the phosphate bond of glucose-1-phosphate depends upon the particular reaction and further varies with the experimental conditions of the same reaction. The acid-catalyzed hydrolysis proceeds through a C$\overset{|}{-}$O—P cleavage. This cleavage is similar to the acid hydrolysis of trimethyl phosphate (5), but opposite to that of carboxylic esters. On the other hand, the incorporation of O^{18} from the medium into the inorganic phosphate during the hydrolysis catalyzed by *acid* phosphatase indicates that the major pathway of the reaction proceeds through the rupture of the C—O$\overset{|}{-}$P bond. The fact that the incorporation of O^{18} was about 20 per cent less than that calculated for this

mechanism may be due to partial hydrolysis by ordinary acid hydrolysis under the conditions of the experiment. This result gives direct evidence of an enzyme actually changing the mechanism of a reaction. Both the enzymatic and non-enzymatic hydrolyses proceed in acid solution and yet the mechanisms are quite different. The C—|—O—P rupture observed in the acid hydrolysis could have resulted from a water reaction (Reaction 1).

$$(1) \quad
\begin{array}{c}
\text{OH} \\
| \\
\text{HO—P}^+\text{—O—|—R} \\
| \\
\text{O}^-
\end{array}
+ \text{OH}_2 \;\rightarrow\;
\begin{array}{c}
\text{OH} \\
| \\
\text{HO—P}^+\text{—O}^- \\
| \\
\text{O}^-
\end{array}
+ \text{ROH}_2^+$$

$$\text{ROH}_2^+ \;\rightarrow\; \text{R—OH} + \text{H}^+$$
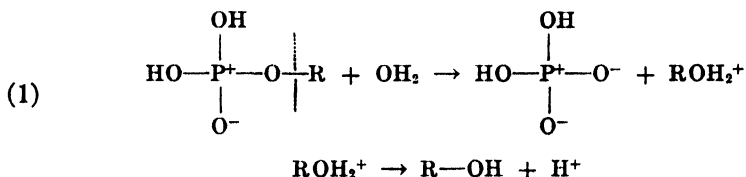
or an acid-catalyzed reaction such as Reaction 2.

$$(2) \quad
\begin{array}{c}
\text{OH} \\
| \\
\text{HO—P}^+\text{—O—R} \\
| \\
\text{O}^-
\end{array}
+ \text{H}^+ \;\rightleftarrows\;
\begin{array}{c}
\text{OH} \\
| \\
\text{HO—P}^+\text{—O—|—R} \\
| \\
\text{OH}
\end{array}
\;\rightarrow\;
\begin{array}{c}
\text{OH} \\
| \\
\text{HO—P}^+\text{—O}^- \\
| \\
\text{OH}
\end{array}
+ \text{R}^+$$

$$\text{R}^+ + \text{H}_2\text{O} \;\rightarrow\; \text{R—OH} + \text{H}^+$$
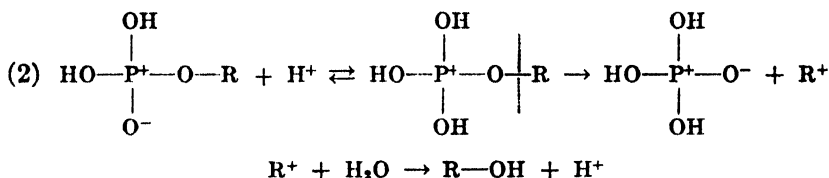
The enzymatic reaction may proceed by the type of acid-catalyzed reaction normally followed by carboxylic esters (Reaction 3).

$$
\begin{array}{c}
\text{OH} \\
| \\
\text{HO—P}^+\text{—O—R} \\
| \\
\text{O}^-
\end{array}
+ \text{H}^+ \;\rightleftarrows\;
\begin{array}{c}
\text{OH} \\
| \\
\text{HO—P}^+\text{—O—R} \\
| \\
\text{OH}
\end{array}
$$

$$(3) \quad
\begin{array}{c}
\text{OH} \\
| \\
\text{HO—P}^+\text{—OR} \\
| \\
\text{OH}
\end{array}
+ \text{H}_2\text{O} \;\rightleftarrows\;
\begin{array}{c}
\text{OH} \\
| \\
\text{HO—P}^+\text{—|—O—R} \\
/ \;\; | \\
\text{HO} \;\; \overset{\text{OH}}{\underset{\text{H}}{}}
\end{array}
\;\rightleftarrows\;
\begin{array}{c}
\text{OH} \\
| \\
\text{HO—P}^+\text{—O}^- \\
| \\
\text{OH}
\end{array}
+ \text{H}^+ + \text{ROH}$$
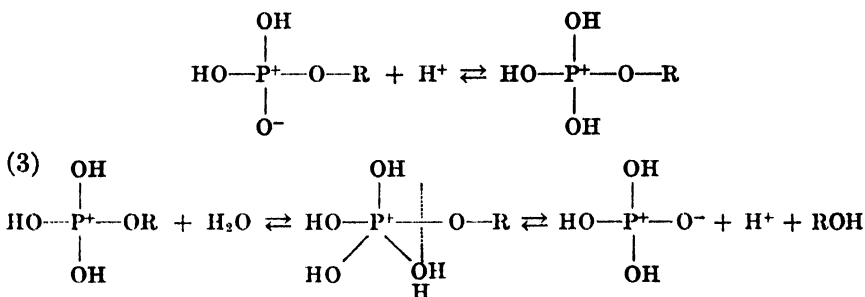
or by a still unknown mechanism.

In the enzymatic hydrolysis catalyzed by alkaline phosphatase, the cleavage also occurs between the oxygen and phosphorus (C—O—|—P). Thus, in so far as the position of bond rupture is concerned, the mechanism which obtains with the phosphatase from intestine acting at pH 9 is the same as the phosphatase from prostate which acts at pH 5. The alkaline phosphatase reaction conforms in mechanism to the non-enzymatic alkaline hydrolysis of phosphates, as shown directly for trimethyl phosphate (4) and indirectly for the phosphate esters of glucose (16). This mechanism

is the same as that found for the alkaline hydrolysis of carboxylic esters (1) and is usually as represented in Reaction 4.

$$OR^- + H_2O \rightarrow HOR + OH^-$$
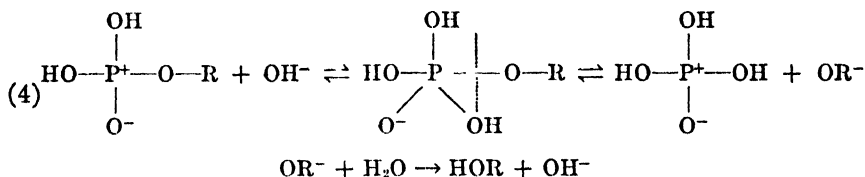
In a study of the mechanism of the muscle phosphorylase reaction, both the rupture of the phosphate bond in glucose-1-phosphate and the rupture of the glucosidic bond of the polysaccharide in the reverse reaction may be determined. If inorganic phosphate is labeled with O^{18} and incubated with glycogen in the presence of the enzyme, and the reaction is allowed to proceed to equilibrium, the O^{18} will be retained by the inorganic phosphate and incorporated without loss in the glucose-1-phosphate only if the forward and reverse reactions do not involve a rupture of the bond between phosphorus and oxygen. On the other hand, if either or both reactions involve a split between phosphorus and oxygen, the O^{18} will be lost to the medium and eventually will be completely equilibrated with the oxygen of the medium. The experimental finding, that the O^{18} content of the inorganic phosphate and glucose-1-phosphate after equilibrium has been reached is the same as the O^{18} concentration of the initial inorganic phosphate, establishes the mechanism for these reactions as given in Reaction 5.
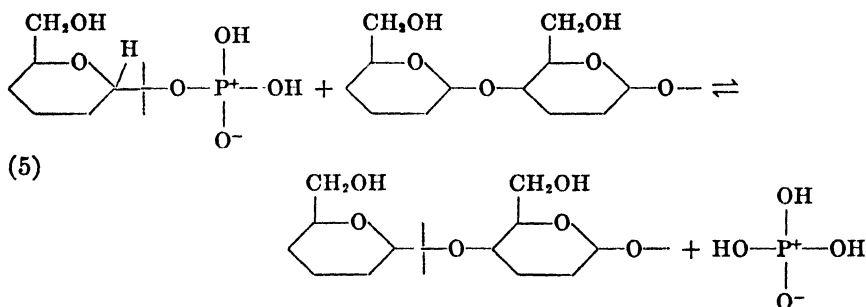
It has previously been demonstrated (13) that the sucrose phosphorylase and muscle phosphorylase enzymes differ fundamentally in their mode of action inasmuch as the former enzyme catalyzes an exchange between the phosphate of glucose-1-phosphate and inorganic phosphate in the absence of an acceptor for the glucose, while the latter enzyme does not. However, the present study indicated that the mechanisms of the two enzymatic reactions do not differ in so far as the rupture of the oxygen bond is con-

cerned. As in the case of the muscle phosphorylase, the mechanism of both the forward and the reverse reactions was determined; namely, the cleavage of glucose-1-phosphate and the cleavage of the glucose-enzyme complex as represented in Reaction 6.

(6)
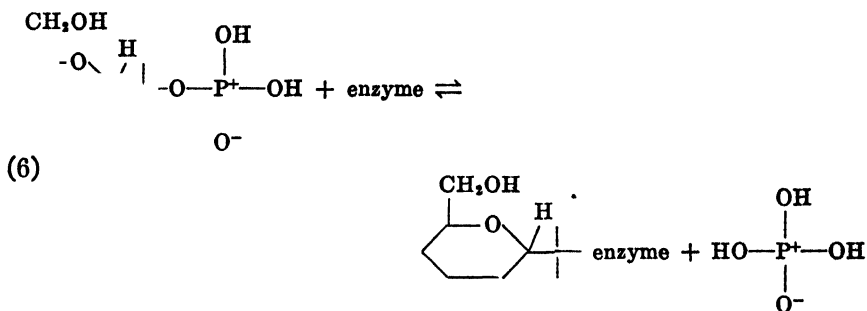
The implications of these mechanisms on the steric course of such reactions are of some interest. In 1927, Robinson (17) suggested that the hydrolysis of phosphoric esters of carbohydrates would occur by the mechanism involving the C$\dashv$O—P split and would therefore be accompanied by a Walden inversion, and furthermore that such a mechanism might account for the biological origin of D-galactose from the hydrolysis of glucose-4-phosphate. Heretofore, experimental verification of this suggestion has been sought by attempts to establish the occurrence or nonoccurrence of a Walden inversion in the hydrolysis of sugar phosphates. No Walden inversion has been found to occur in alkaline hydrolysis (16) in which the split is probably C—O$\dashv$P. In the hydrolysis of glucose-1-phosphate by acid and alkaline phosphatase, the rupture has been shown to be C—O$\dashv$P and no inversion would occur.

However, the question arises as to whether a reaction involving a C$\dashv$O—P cleavage must necessarily be accompanied by a Walden inversion. In the present study, the C$\dashv$O—P split has been found to occur in three reactions of glucose-1-phosphate, the acid-catalyzed hydrolysis, the muscle phosphorylase reaction, and in the sucrose phosphorylase reaction. In these two enzymatic reactions which proceed by a C$\dashv$O—P cleavage, it is definitely known that no inversion takes place. In the muscle phosphorylase reaction, the reactant is α-glucose-1-phosphate and the polysaccharide end-product contains only α-glucose units. Similarly in the sucrose phosphorylase reaction, there is no change in configuration from α-glucose-1-phosphate to sucrose. Undoubtedly the participation of the enzyme in the reaction introduces steric constraints which maintain configuration in spite of conditions which would ordinarily lead to inversion.

In the acid-catalyzed hydrolysis reaction involving the rupture of a C$\dotplus$O bond, one might expect inversion to occur;[2] unfortunately, the rate of mutarotation is so rapid under the conditions of hydrolysis that it is impossible to establish the occurrence of inversion experimentally. Whether the results obtained with glucose-1-phosphate can be extended to other phosphate esters remains for further investigation. Glucose-1-phosphate is by no means a typical ester structurally and this fact is reflected in its relative lability towards acid hydrolysis and stability towards alkaline hydrolysis. However, if the acid hydrolysis of glucose-3-phosphate, for example, proceeds by the same mechanism, one should probably be able to demonstrate inversion or loss of configuration. Should the enzymatic hydrolysis mechanisms found for glucose-1-phosphate with C—O$\dotplus$P cleavage prove to be universal, there could be no inversion induced by phosphatase action.

SUMMARY

The mechanisms of several reactions involving the cleavage of glucose-1-phosphate have been investigated with O^{18}. A method was devised for analyzing phosphate for its isotopic oxygen content. It was found that, in acid hydrolysis, the rupture occurred between the carbon of glucose and oxygen but, in hydrolysis catalyzed by acid phosphatase or alkaline phosphatase, the bond between oxygen and phosphorus is ruptured. In the muscle phosphorylase and sucrose phosphorylase reactions, the rupture of the bond occurs between carbon and oxygen of glucose-1-phosphate in the dephosphorylation reaction and the same bond rupture is involved in the reverse reactions. Some of the implications of these cleavages on the mechanisms of the reactions are discussed.

BIBLIOGRAPHY

1. Polanyi, M., and Szabo, A. L., *Tr. Faraday Soc.*, **30**, 508 (1934).
2. Datta, S. C., Day, J. N. E., and Ingold, C. K., *J. Chem. Soc.*, 838 (1939).
3. Roberts, I., and Urey, H. C., *J. Am. Chem. Soc.*, **60**, 2391 (1938).
4. Herbert, J. B. M., and Blumenthal, E., *Nature*, **144**, 248 (1939).
5. Blumenthal, E., and Herbert, J. B. M., *Tr. Faraday Soc.*, **41**, 611 (1945).
6. Bentley, R. M., Abstracts, American Chemical Society, Atlantic City, Apr. (1947).

[2] If the water reaction predominates, inversion at carbon 1 should result, while the hydrogen ion-catalyzed reaction should lead to loss of configuration on carbon 1 with consequent partial racemization.

7. Cohn, M., and Urey, H. C., *J. Am. Chem. Soc.*, **60, 679** (1938).
8. Nier, A. O., *Rev. Scient. Instruments*, **18, 398** (1947).
9. Blumenthal, E., and Herbert, J. B. M., *Tr. Faraday Soc.*, **33, 849** (1937).
10. Winter, E. R. S., Carlton, M., and Briscoe, H. V. A., *J. Chem. Soc.*, **131** (1940); 631 (1942).
11. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66, 375** (1925).
12. Green, A. A., and Cori, G. T., *J. Biol. Chem.*, **151, 21** (1943).
13. Cohn, M., and Cori, G. T., *J. Biol. Chem.*, **175, 89** (1948).
14. Doudoroff, M., Barker, H. A., and Hassid, W. Z., *J. Biol. Chem.*, **168, 725** (1947).
15. Doudoroff, *J. Biol. Chem.*, **151, 351** (1943).
16. Percival, E. E., and Percival, E. G. V., *J. Chem. Soc.*, 874 (1945).
17. Robinson, R., *Nature*, **120, 44** (1927).

THE CONVERSION OF METHIONINE TO CYSTINE IN A HUMAN CYSTINURIC*

By LESTER J. REED, DORIANO CAVALLINI,† FRED PLUM, JULIAN R. RACHELE, AND VINCENT DU VIGNEAUD

(From the Department of Biochemistry, and the Department of Medicine, Cornell University Medical College, and The New York Hospital, New York City)

The opportunity of studying sulfur metabolism in a human cystinuric by the tracer technique with radioactive sulfur was presented to us recently. As a means of exploring the feasibility of this approach it was decided to extend to the human cystinuric the type of experiment already carried out upon canine cystinurics with radiomethionine; namely, the tracing of the sulfur of administered methionine to the excreted cystine by means of radiosulfur (1).

Before administering the radioactive methionine to the patient considerable care was taken to assure us that the dosage employed was well within the tolerance suggested by Morgan and by Marinelli, Quimby, and Hine (2, 3). In addition, it was decided to employ our preparation of radiomethionine first on cystinuric dogs, which had been generously placed at our disposal by Dr. Erwin Brand. Our results on dogs agreed with those reported by Tarver and Schmidt (1) and the details of this experiment will therefore not be included in the present communication.

The patient, a 24 year-old white woman weighing 50 kilos, was fed 200 mg. of methionine containing 2.40×10^7 counts per minute[1] of S^{35}. It was calculated (3) that this dosage produced approximately 0.01 equivalent roentgen per day. This was considered well within the limits of safety, since the maximum safe daily radiation tolerance is 0.1 equivalent roentgen (2, 3). For a period of 6 days before and 5 days after the feeding of the radioactive methionine, the patient was kept on a low protein diet (50 gm. per day). A slight transitory fall in hemoglobin and red blood count and a fleeting manifestation of increased capillary fragility were observed. Throughout the course of the experiment the patient was closely observed. She was followed in the hospital for 10 days, then

---

[1] All the counts reported in this paper are the counts measured on the Geiger-Müller counter and corrected for background and self-absorption. They represent only a fraction of the absolute radioactivities of the samples.

as an outpatient at weekly intervals for 2 months, and at less frequent intervals for an additional period of 18 months. She will be continued under further observation. No ill effects were noted that could be attributed to the isotope.

The distribution of the sulfur in the urine as total sulfate, inorganic and organic sulfate, and cystine sulfur was determined daily for 4 days following the feeding of the radioactive methionine and the radioactivity of these various sulfur fractions was measured. For the determination of the radioactivity of the cystine fraction, the amino acid was isolated from the urine samples for the 4 days following the methionine feeding by the "washing out" technique. The total radioactivity in the urine and in the blood was measured at longer intervals after the feeding of the methionine for a period of 70 days. To obtain an index of the radiosulfur of the cystine of the tissues of the body, the axillary hair was clipped at the time of the feeding of the labeled methionine and again 20 days later, and the radioactivity of the cystine isolated from the latter sample was determined.

<div align="center">EXPERIMENTAL</div>

Synthesis of Methionine—124 mg. of radioactive barium sulfate were converted to benzyl β-chloroethyl sulfide (crude) in 82 per cent yield according to the directions of du Vigneaud and coworkers (4). The latter compound was diluted with 1.06 gm. of unlabeled benzyl β-chloroethyl sulfide and converted to S-benzyl-DL-homocysteine in 63 per cent yield according to the directions of Kilmer and du Vigneaud (5). The labeled benzylhomocysteine was converted to analytically pure methionine in 74 per cent yield by the method of Patterson and du Vigneaud (6). The over-all yield of analytically pure methionine from barium sulfate was 38 per cent. The methionine had an activity of 5.57×10^5 counts per minute per mg. of S on the day it was administered to the patient.

Urine Analysis—The urine was filtered through a layer of Hyflo Super-Cel and the latter was washed with about 50 ml. of 1 N hydrochloric acid. The acid extract was added to the main filtrate. The washed Super-Cel did not contain any radioactive material.

A 200 ml. aliquot of the 24 hour specimen was freed of phosphate (1, 7) by treating with 20 ml. of 12.5 per cent ammonium chloride, sufficient ammonium hydroxide to make the solution alkaline to phenolphthalein, and 3 gm. of magnesium carbonate. The mixture was diluted to 250 ml., shaken, and filtered after being allowed to stand for 1 hour.

The total sulfur was determined in duplicate on 10 ml. aliquots of the filtrate by digesting with 10 ml. of Pirie's reagent (8). The digestion was carried out in a 100 ml. Vycor evaporating dish on a sand bath. When the contents were dry (2 to 3 hours) the dish was slowly brought to red heat

with a Meker burner and allowed to cool for 1 minute. Then 2 ml. of Pirie's reagent were added and the contents were brought to dryness again and ignited. The black residue was dissolved in 6 ml. of 6 N hydrochloric acid and reevaporated to dryness. The brown residue was dissolved in 20 ml. of water and the sulfur content was determined by titration of the benzidine sulfate precipitate according to Fiske (7). However, determination of the sulfur by precipitation of the sulfate as barium sulfate, followed by ignition and weighing (9), gave values which were consistently

TABLE I

Sulfur and Radioactivity Partitions in Urine of Cystinuric Given Labeled Methionine

	Day 1		Day 2		Day 3		Day 4	
	Sulfur	Per cent of total	Sulfur	Per cent of total	Sulfur	Per cent of total	Sulfur	Per cent of total
Sulfur partition								
	mg		*mg.*		*mg.*		*mg.*	
Total sulfur	189.0	100	308.0	100	434.0	100	315.5	100
" sulfate	87.8	46	159.0	52	208.5	48	160.0	51
Neutral sulfur	101.2	54	149.0	48	225.5	52	155.5	49
Cystine	37.6	20	95.2	31	152.0	35	100.4	32
Inorganic sulfate	54.9	29	137.0	44	183.0	42	144.0	46

	Radioactivity partition							
	Counts per min. per mg S	Per cent of total administered radio-activity	Counts per min. per mg. S	Per cent of total administered radio-activity	Counts per min. per mg. S	Per cent of total administered radio-activity	Counts per min. per mg. S	Per cent of total administered radio-activity
Total sulfur	16,900	13.30	1850	2.38	1000	1.80	693	0.90
" sulfate	2,055	0.74	1210	0.82	598	0.53	422	0.29
Neutral sulfur	29,700	12.54	2535	1.56	1372	1.27	972	0.61
Cystine	4,180	0.66	1990	0.78	1260	0.78	950	0.40
Inorganic sulfate	2,080	0.49	1295	0.74	613	0.45	430	0.25

higher than those obtained by the Fiske method (compare with Pirie (8)). The values reported in Table I are those obtained by gravimetric determination as barium sulfate.

The total sulfate was determined in duplicate on 25 ml. portions of the phosphate-free urine filtrate. The sample was first hydrolyzed by adding 5 ml. of 3 N hydrochloric acid and evaporating the solution to dryness on a steam bath. The black residue was stirred with 50 ml. of water and filtered. 20 ml. of this filtrate were used for sulfate determination by titration of the benzidine sulfate precipitate according to Fiske (7). Inorganic sulfate determinations were made in duplicate on 10 ml. portions

of the urine filtrate by the method of Fiske. The values are recorded in Table I. Gravimetric determinations of inorganic and total sulfate as barium sulfate gave values in close agreement with the values obtained by the titration method.

Cystine was determined on a 25 ml. portion of the original Super-Cel filtrate. The cystine was precipitated as the cuprous mercaptide according to the method of Brand and coworkers (10), and the mercaptide was decomposed with hydrogen sulfide. The filtrate from the sulfide precipitation was concentrated to the original volume of the urine sample (11) and the cystine was determined by the Sullivan method (12), with a cystine standard. The values recorded in Table I are those carried out on samples that had been stored for 2 months in the refrigerator. The cystine content of a cystine standard, which had been stored with the urinary cystine samples, was found to be unchanged.

Isolation of Cystine from Urine—To 400 ml. of urine were added 50 mg. of unlabeled L-cystine dissolved in dilute hydrochloric acid. The cystine was precipitated as the cuprous mercaptide and the latter was decomposed with hydrogen sulfide. The filtrate from the hydrogen sulfide treatment was evaporated to dryness under reduced pressure. The residue was dissolved in 10 ml. of 1 N hydrochloric acid, treated with norit, and filtered. A dilute alcoholic solution of iodine was added to the filtrate until a test with sodium nitroprusside was negative. Cystine was separated by isoelectric precipitation, redissolved in 0.5 N hydrochloric acid, and reprecipitated with ammonia. The samples were recrystallized three or four times. The sulfur content of these samples differed by less than 1 per cent from the theoretical values. Occasionally samples of cystine contained traces of copper which were removed by retreatment with hydrogen sulfide followed by recrystallization of the cystine. From the radioactivity of these recrystallized cystine samples, it was possible to calculate the radioactivity of the cystine in the urine samples. These latter values are recorded in Table I.

Isolation of Cystine from Hair—To the axillary hair (31 mg.) were added 30 mg. of unlabeled L-cystine. The mixture was heated under a reflux for 20 hours with 8 ml. of a 1:1 hydrochloric acid-formic acid mixture (13). Cystine was isolated as the cuprous mercaptide (13, 14), which was treated as described in the procedure for the isolation of cystine from urine. From the radioactivity of this cystine sample, the radioactivity of the sulfur in the axillary hair was determined. The value is recorded in Table II.

Determination of Radioactivity—Radioactivity determinations on the total sulfur, organic sulfate, and the inorganic sulfate fractions of the urine

were made on the same samples used for sulfate determinations by the method involving titration of the benzidine sulfate precipitate. The benzidine was removed by filtration and the sulfate was precipitated as benzidine sulfate from aliquots of the filtrate containing 1 mg. of sulfur. The cystine samples isolated from the urine were oxidized by the Carius method and the sulfate was precipitated as benzidine sulfate. For the determination of the total radioactivity in the urine samples for the 13th, 20th, 40th, and 70th day, the samples were oxidized with Pirie's reagent and the sulfur was precipitated as benzidine sulfate. The precipitates were filtered and collected by the method described by Henriques and coworkers (15).

The radioactivity measurements were made with the use of a thin mica window (2 mg. per sq. cm.) bell-shaped Geiger-Müller counter (15)

TABLE II

Radioactivity Measurements of Urine, Blood, and Hair

Day	Urine			Blood	Hair
	Counts per min. per mg. S	Total sulfur	Per cent of total administered radioactivity	Counts per min. per ml.	Counts per min. per mg. S
		mg.			
13	294	488	0.67		
20	255	574	0.72	615	703
40	103	632	0.38	474	
70	56	610	0.25	271	

and a scaling circuit. The radioactivity values were corrected for background and self-absorption (16, 17).

Blood—A 2 ml. oxalated blood sample was oxidized according to the method of Stockholm and Koch (18). The sulfate was precipitated as barium sulfate, which was collected for radioactivity determinations by the same procedure as was used for the benzidine sulfate. The radioactivity values for blood samples taken on the 20th, 40th, and 70th day after the feeding of the radioactive methionine are given in Table II.

Non-Identity with Methionine of Radioactive Material in Neutral Sulfur Fraction—The method of paper chromatography as applied to biological fluids by Dent (19) was employed in preliminary experiments. The urine sample (0.1 ml.) was placed evenly across a strip of No. 1 Whatman filter paper about 6 cm. from the end which was dipped into the solvent, and the latter was allowed to flow down over the paper. The paper was dried, developed with ninhydrin, and cut into sections. Each section was placed under the mica window of a Geiger-Müller counter to determine

activity. When a 0.1 ml. urine sample containing 25 γ of added **methionine** was chromatographed with collidine (saturated with water) as the solvent, the active material was located near the point of application of the sample, R_F about 0.05, whereas the methionine traveled farther down the strip (R_F 0.45). A similar separation of added methionine and the active material in the urine was effected with use of butanol (saturated with water) as the solvent. The activity on the strip was located at the point of application of the sample; methionine R_F 0.11. When phenol (saturated with water) was used as the solvent, the active material moved approximately 0.85 of the total distance traveled by the solvent. The strip showed no color at this point after being developed with ninhydrin.

<div align="center">RESULTS AND DISCUSSION</div>

These data demonstrate the incorporation of the sulfur of ingested methionine into tissue and urinary cystine in a human cystinuric. This supports the findings of Brand and others (20–22).

During a period of 4 days after the ingestion of the labeled methionine, 2.6 per cent of the radiosulfur was excreted in the form of cystine. A total of 18.4 per cent of the administered radiosulfur was excreted during these 4 days, 13.30 per cent being excreted during the 1st day after the feeding of the radioactive methionine. There was a slow loss of isotope into the urine during the following 2 months.

The results are comparable with those obtained in work with cystinuric dogs. Tarver and Schmidt (1) administered radioactive methionine intravenously and subcutaneously to two cystinuric dogs. 6.11 and 12.23 per cent, respectively, of the radioactive sulfur of the administered methionine was excreted during a period of 4 days, 0.18 and 0.31 per cent, respectively, in the form of cystine. The percentages of the radiosulfur excreted during the 1st day after administration of the radioactive methionine were 2.78 and 6.93 per cent, respectively. In experiments conducted in this laboratory with two cystinuric dogs, it was found that 11.8 and 16.1 per cent, respectively, of the radiosulfur of ingested methionine was excreted during a 4 day period, 0.37 and 1.60 per cent, respectively, being present in excreted cystine.

As in the work with cystinuric dogs it was found that the inorganic sulfate and the total sulfate fractions from the urine of the human cystinuric had very nearly the same radioactivity. Also, the activity of the neutral sulfur fraction was greater than that of the cystine sulfur. Experiments with paper chromatograms indicated that this additional activity in the neutral sulfur fraction was not due to appreciable methionine. It is hoped that this aspect of the problem will be explored further.

SUMMARY

Radioactive methionine, containing approximately 100 μc. of S^{35}, was fed to a human cystinuric without harmful effects. Cystine was isolated from the urine and from newly grown hair and was found to be radioactive.

Most of the radioactive sulfur (81.6 per cent) was retained by the subject during a period of 4 days, only 18.4 per cent being excreted. 2.6 per cent of the administered radiosulfur was excreted in the form of cystine during the first 4 days after the administration of the methionine. During the 1st day after the feeding of the radioactive methionine, 13.3 per cent of the radioactive sulfur was excreted. There was a slow loss of isotope into the urine for a period of 70 days, at which time the experiment was terminated.

The radioactivity of the neutral sulfur fraction of the urine was greater than that of the cystine sulfur. Preliminary experiments with paper chromatograms have indicated that this additional activity was not due to excreted methionine.

The results are compared with those obtained in experiments with cystinuric dogs.

BIBLIOGRAPHY

1. Tarver, H., and Schmidt, C. L. A., *J. Biol. Chem.*, **167**, 387 (1947).
2. Morgan, K. Z., *J. Phys. and Colloid Chem.*, **51**, 984 (1947).
3. Marinelli, L. D., Quimby, E. H., and Hine, G. J., *Am. J. Roentgenol.*, **59**, 260 (1948).
4. Wood, J. L., Rachele, J. R., Stevens, C. M., Carpenter, F. H., and du Vigneaud, V., *J. Am. Chem. Soc.*, **70**, 2547 (1948).
5. Kilmer, G. W., and du Vigneaud, V , *J. Biol. Chem.*, **154**, 247 (1944).
6. Patterson, W. I., and du Vigneaud, V., *J. Biol Chem.*, **123**, 327 (1938).
7. Fiske, C. H., *J. Biol. Chem.*, **47**, 59 (1921).
8. Pirie, N. W., *Biochem. J.*, **26**, 2044 (1932).
9. Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical physiological chemistry, Philadelphia, 12th edition, 887 (1947).
10. Brand, E., Cahill, G. F., and Kassell, B., *J. Biol. Chem.*, **133**, 431 (1940).
11. Hess, W. C., and Sullivan, M. X., *J. Biol. Chem.*, **143**, 545 (1942).
12. Sullivan, M. X., and Hess, W. C., *J. Biol. Chem.*, **116**, 221 (1936).
13. du Vigneaud, V., Kilmer, G. W., Rachele, J R., and Cohn, M., *J. Biol. Chem.* **155**, 645 (1944).
14. Graff, S., Maculla, E., and Graff, A. M., *J. Biol. Chem.*, **121**, 81 (1937).
15. Henriques, F. C., Jr., Kistiakowsky, G. B., Margnetti, C., and Schneider, W. G., *Ind. and Eng. Chem., Anal. Ed.*, **18**, 349 (1946).

16. Libby, W. F., *Ind. and Eng. Chem., Anal. Ed.*, **19**, 2 (1947).
17. Reid, A. F., in Wilson, D. W., Preparation and measurement of isotopic tracers, Ann Arbor, 83 (1946).
18. Stockholm, M., and Koch, F. C., *J. Am. Chem. Soc.*, **45**, 1953 (1923).
19. Dent, C. E., *Lancet*, **2**, 637 (1946).
20. Brand, E., Cahill, G. F., and Harris, M. M., *J. Biol. Chem.*, **109**, 69 (1935).
21. Brand, E., Block, R. J., Kassell, B., and Cahill, G. F., *J. Biol. Chem.*, **119**, 669 (1937).
22. Lewis, H. B., Brown, B. H., and White, F. R., *J. Biol. Chem.*, **114**, 171 (1936).

THE HEXOKINASE OF THE RAT ERYTHROCYTE AND THE INFLUENCE OF HORMONAL AND OTHER FACTORS ON ITS ACTIVITY*

By WILLIAM R. CHRISTENSEN, CALVIN H. PLIMPTON, AND ERIC G. BALL

WITH THE TECHNICAL ASSISTANCE OF DOROTHY HUGHES

(*From the Department of Biological Chemistry, Harvard Medical School, Boston*)

An impetus to the study of the interaction of hormones and enzymes has been given by the recent investigations reported by Cori and coworkers (3, 5, 14, 15). Their findings suggest that insulin, a pituitary hormone, and a hormone from the adrenal cortex are intimately involved in carbohydrate metabolism, in part at least, through their controlling action on the activity of hexokinase. This enzyme catalyzes the formation of hexose-6-phosphate from glucose and adenosine triphosphate, a reaction which is the initial step in glucose utilization by the living cell. Now unfortunately, the investigation of this system of interacting components *in vitro* is complicated by the lability of several of the actors, especially so in the crude tissue extracts which have served as a source of the enzyme. As a consequence, we find that a reproducibility of results from experiment to experiment or from laboratory to laboratory has not been achieved (2, 12, 16, 19).

It occurred to us that the use of the erythrocyte as a source of the enzyme system might eliminate some of the uncertainties attendant upon employment of other tissue extracts. The presence in the red blood cell of hexokinase was first indicated by Meyerhof (11), and has been most recently confirmed by Speck and Evans (18). In addition, it is well known that glucose is converted quantitatively to lactic acid in the red cell and that enzymes for the conversion of glucose to glycogen or the further metabolism of lactate appear to be absent. Thus, the erythrocyte would appear to be the simplest mammalian cell available for a study of hexokinase activity.

We present here investigations on the hexokinase activity of hemolysates of erythrocytes obtained from normal, diabetic, and hypophysectomized rats along with observations on the effect of various hormone preparations upon them.

As has been previously indicated, earlier studies have clearly demonstrated the presence of hexokinase activity in red cell hemolysates. Our chief concern, therefore, was first the development of a reproducible system of maximum activity. The nutritional status of the animal, the effect of anticoagulants, the presence or absence of plasma (represented by the number of times the red cells were washed), the duration of hemolysis, the temperature at which the washing and hemolysis were performed, the effect of fluoride and iodoacetate upon the final reaction, and the ideal concentration of other substances required in the final manometric reaction mixture were investigated as factors that might affect the rate and specificity of the reaction. These studies led to the establishment of the following routine method of preparation.

Only rats of the Wistar or Sprague-Dawley strains have been employed. Blood was obtained by cardiac puncture and withdrawn into a syringe containing 0.1 ml. of heparin as an anticoagulant. After inversion of the syringe to mix the blood and heparin, its contents were discharged into a centrifuge tube immersed in an ice bath. A volume of cold isotonic saline equal to 3 times the original blood volume was added and the mixture thoroughly agitated. Centrifugation was performed in a 5° cold room; the clear supernatant was removed and the same washing process repeated twice. After removal of the supernatant of the third washing, the red cells were hemolyzed by the addition of twice their volume of cold distilled water. A period of 15 minutes was allowed for this operation which was carried out at a temperature of $-2°$. The material was then centrifuged at high speed in the cold to remove the cell stroma and the crystalline hemoglobin which precipitated as a result of this treatment. The final supernatant represents the preparation used. Although the amount of hemoglobin which it contained varied from preparation to preparation (influenced by the exact temperature of the bath and oxygen saturation of the hemoglobin), the major portion was removed in most instances, leaving a slightly pink tinged solution. Enzyme activity was determined manometrically according to the technique of Colowick and Kalckar (4). In these studies of activity, the Warburg vessels were set up as in Table I unless otherwise noted.

Adenosine triphosphate (ATP) was prepared by us from fresh rabbit muscle according to the techniques described by Lohman (10) and by LePage (9). Its purity, as judged by the content of hydrolyzable phosphorus, varied between 85 and 90 per cent.

Determinations were carried out at 37.5°. The flasks were equilibrated with 95 per cent N_2-5 per cent CO_2 for a period of 10 minutes, the cocks were then closed, and the manometers read until a steady state had been

attained, usually 10 minutes. Thereafter, the contents of the side bulbs were tipped and the CO_2 evolution measured at 10 minute intervals for a total of 1 hour.

Since we are dealing primarily with a comparison of rates of reactions, all results are expressed as c.mm. of CO_2 evolved during the 1st hour of the reaction only. As can be seen from the data presented in Fig. 1, the rate of reaction begins to decline towards the end of the 1st hour period.

In order to determine whether or not phosphorus actually was being transferred from ATP to glucose with the formation of glucose-6-phosphate, adenosine diphosphate (ADP), and the evolution of stoichiometric quantities of carbon dioxide, a series of partition studies was performed. Glucose,

TABLE I

Contents of Warburg Vessels

	Reagent	Concentration	Amount	Total final concentration after mixing
			ml.	
Main compartment	NaHCO₃	0.2 M	0.5	0.033 M
	NaF	0.5 "	0.2	0.033 "
	Glucose	2%	0.1	66 mg.%
	Hexokinase preparation		1.0*	
Side bulb	ATP	0.04 M	0.2	0.0026 M
	NaHCO₃	0.1 "	0.05	
	MgCl₂	0.2 "		0.0033 "

Water added to final volume of 3.0 ml.

ATP added as neutral sodium salt. Final pH of gas-equilibrated mixture 7.5.
* Equal to content of 0.33 ml. of packed red cells.

inorganic phosphorus, readily hydrolyzable phosphorus, and total phosphorus concentration were determined in the reaction mixture initially and after various periods of activity during which time CO_2 evolution was measured. Glucose was determined by the Nelson method (13) with the $Ba(OH)_2$-$ZnSO_4$ precipitation technique described by Somogyi (17). A 1 ml. aliquot was used. It has been shown that glucose-6-phosphate is eliminated as a reducing substance in this technique (3). Phosphorus determinations were done according to the method of Fiske and Subbarow (7) on trichloroacetic acid filtrates. Easily hydrolyzable phosphorus was considered to be that released by 7 minutes exposure at 100° in 1 N HCl. The results of these partition studies are presented in Table II. Preliminary studies comprising the addition of known amounts of acid to the complete reaction mixture had indicated that no significant corrections were necessary for the retention of CO_2.

Examination of these data reveals that the reaction is characterized by a decrease in hydrolyzable phosphorus and an increase in inorganic plus organic phosphorus which is almost exactly equivalent. The major portion of the hydrolyzable phosphorus which disappears is represented by an increase in organic phosphorus, signifying a transfer of this element from ATP to glucose. The CO_2 produced and the decrease in hydrolyzable phosphorus are roughly equivalent to each other but somewhat greater than the increase in organic phosphorus and utilization of glucose. The latter is definitely smaller than the decrease in hydrolyzable phosphorus.
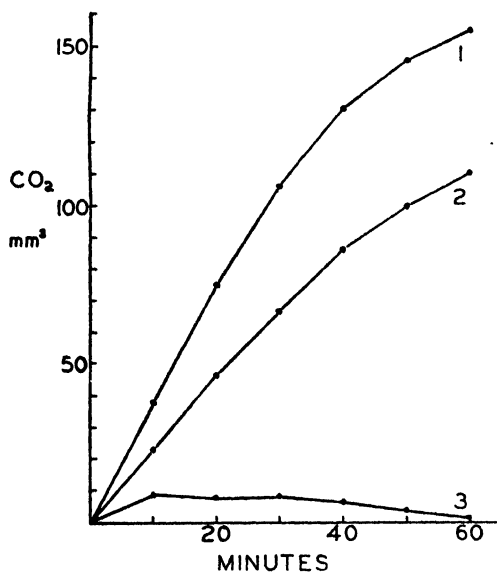
Fig. 1. Manometric time course of hexokinase reaction. Reaction mixture as described in text except as noted. Curve 1, fluoride omitted; Curve 2, all components present; Curve 3, glucose omitted.

However, if the increase in inorganic phosphorus is subtracted from the decrease in hydrolyzable, the resultant value is fairly close to the increase in organic phosphorus and glucose utilization. On the basis of this evidence, it appears logical to assume that the major reaction is a phosphorylation of the glucose and that the evolution of CO_2 is a reasonable index to the decrease in hydrolyzable phosphorus and the disappearance of glucose. A significant amount of inorganic phosphorus is produced in the reaction. It is impossible to say whether this is due to hydrolysis of ATP or of glucose-6-phosphate. In the two instances in Table II in which the reaction was allowed to proceed to equilibrium, only one-half of the available hydrolyzable phosphorus had been utilized. This may be taken as

further proof of the specificity of the reaction, indicating that all ATP had been converted to ADP and that the reaction had proceeded no further. Also, as can be seen from the data given in Table II, in the absence of fluoride phosphatase activity is uninhibited and, as a consequence, large amounts of inorganic phosphorus and CO_2 appear, but little organic phosphorus. Furthermore, in the absence of the normal substrate, glucose, little or no change occurs. The small amount of organic phosphorus appearing can be attributed to the presence of small amounts of glucose in the hemolysate. A detectable concentration was always present despite the washing of the cells.

TABLE II

Changes in Phosphorus, Glucose, and Carbon Dioxide during Manometric Determination of Hexokinase Activity

All values expressed in micromoles.

	Experiment No.	Decrease in hydrolyzable P	Increase in inorganic P	Increase in organic P	CO_2 produced	Glucose utilized	Stage of reaction
Normal reaction	1	5.83	1.87	4.62	5.72		Equilibrium
	2	6.84	2.03	4.55	5.67		"
	3	5.90	1.06	4.75	4.95	3.34	1 hr.
	4	4.07	0.90	3.16	4.28	3.72	1 "
	5	3.45	0.74	2.71	4.36	3.27	1 "
	6	5.61	1.70	4.73	4.80		1 "
Average		5.28	1.38	4.09	4.96	3.44	
Normal reaction without fluoride	3b	6.94	4.97	1.74	6.97		1 hr.
Normal reaction without glucose addition	3c	1.94	1.42	0.48	0.31		1 "

The kinetics of the normal reaction are shown in Fig. 1. As would be expected from the partition data, an excess of CO_2 is produced in the absence of fluoride and in the absence of glucose essentially no reaction occurs. The decrease in the rate of reaction in the complete system over a period of time can be attributed to the decrease in concentration of ATP. If followed long enough, an equilibrium state is eventually achieved. As emphasized previously, it is the rate of the reaction in which we are interested. Therefore, readings during the 1st hour of the reaction are of the greatest value for our studies.

The activity of the enzyme preparation on various monosaccharide substrates is presented in Fig. 2. Maximum activity is obtained in the

presence of glucose, followed by mannose and fructose in order of decreasing activity. The enzyme apparently does not phosphorylate galactose, since the base-line activity of the enzyme preparation is unaffected by the addition of this substrate. The fact that the activity is no greater in the presence of both glucose and fructose than in the presence of glucose alone may be taken to indicate that the same enzyme acts upon glucose and fructose. Using the rates of reaction for the 30 to 60 minute period shown in Fig. 2, we may calculate that the relative rates of reaction of glucose, mannose, and fructose are as $1.0:0.77:0.36$. Apparently, the characteristics of this enzyme are considerably different in respect to its activity
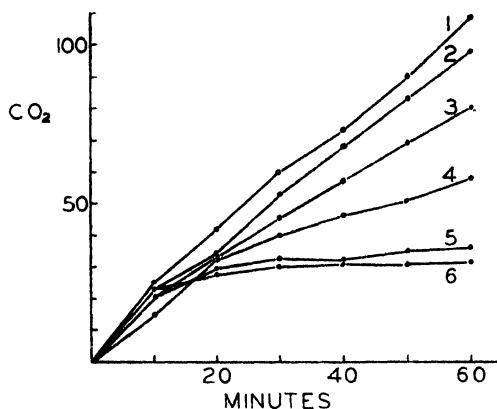
FIG. 2. Hexokinase activity with various hexoses as substrates. Curve 1, glucose final concentration 66 mg. per cent; Curve 2, glucose, final concentration 33 mg. per cent + fructose, final concentration 33 mg. per cent; Curve 3, mannose, final concentration 66 mg. per cent; Curve 4, fructose, final concentration 66 mg. per cent; Curve 5, galactose, final concentration 66 mg per cent; Curve 6, no hexose added.

upon various monosaccharides from that obtained from yeast or from other animal tissues (1, 6, 8).

During the preliminary stages of this investigation, it was observed that the enzyme activity of hemolysates varied with the number of times the red cells were washed. This suggested that plasma had an inhibiting effect upon the enzyme activity, which proved to be the case. The magnitude of this inhibition is illustrated in Fig. 3. This same effect was reproduced by the addition of serum from either clotted or defibrinated rat blood. The inhibiting agent was destroyed by boiling. It was repeatedly demonstrated, however, that after three washings of the erythrocytes, maximum enzyme activity in the hemolysates was achieved and further cell washings were without effect. That inhibition by plasma is not due to a non-specific protein effect such as a buffering action is shown by the

fact that the addition of purified serum albumin or crystalline hemoglobin in comparable amounts caused no similar retardation of the reaction.
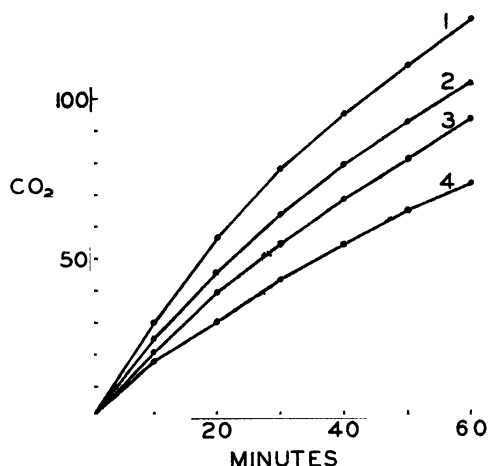
Fig. 3. Hexokinase activity of hemolysates from rat red blood cells as affected by plasma.   Curve 1, hemolysate from cells washed three times; Curve 2, hemolysate as in Curve 1 plus 0.2 ml. of rat plasma; Curve 3, hemolysate as in Curve 1 plus 0.4 ml. of rat plasma; Curve 4, hemolysate from unwashed cells.

TABLE III

Effect of Cations on Hexokinase Activity

The activity values given are the average of duplicate analyses for the 1st hour of the reaction.   The cation concentrations were calculated from the amount and strength of reagents added; the red blood cell hemolysate was assumed to yield K equivalent to a final concentration of 0.012 M.   The experimental procedure was as described in the text, except that NaCl or KCl was added to those vessels with the highest salt content, and in those vessels with high K content all reagents except ATP were added as the potassium salts

Final molar concentration			Activity, c.mm. CO_2 per hr.	
Na	K	Na + K	Experiment 1	Experiment 2
0.077	0.012	0.089	117	102
0:148	0.012	0.160	78	80
0.010	0.079	0.089	91	90
0.010	0.150	0.160	67	75

Another factor that was found to influence the rate of reaction was the cation composition of the test system (*cf.* Table III).   If NaCl or KCl were added to the test system in quantities sufficient to raise the total ionic strength to that encountered physiologically, then the activity was

appreciably lowered. Also, a shift from a medium with sodium as the predominant cation to one mainly potassium caused a decrease in activity. This latter effect was, however, more noticeable at lower total salt concentrations. No studies of variations in the anion composition of the media were made.

With these basic studies to guide us, we next proceeded to the investigation of hexokinase activity in various abnormal physiological states. A necessary preliminary to this, however, was the establishment of a baseline level for the normal rat. In a series of thirty-two normal rats, in

TABLE IV

Effect of Insulin and Adrenal Cortical Extract on Hexokinase from Normal Rat Erythrocytes

All values are the average of duplicates and are expressed as c.mm. of CO_2 per hour. The experimental conditions were as described in the text, except for the addition of the hormones to the main compartment of the vessels.

Experiment No	No addition	With insulin*	With ACE†
	Hemolysate of washed cells		
1	96	95	92
2	110	110	107
3	106	103	105
4	117	112	115
	Hemolysate of unwashed cells		
5	66	64	62
6	85	86	83
7	64	66	62
8	47	49	51

* 100 γ per vessel; Lilly, crystalline.
† 0.1 ml. per vessel; Upjohn, alcoholic extract.

which no selection was made other than that previously mentioned, a mean value for the hexokinase activity of 97 c.mm. of CO_2 per hour was obtained under the test conditions previously outlined. The standard deviation was ±12.25. These values serve to indicate that the hexokinase activity was fairly constant from rat to rat, showing a typical biological scatter. In all later studies, it is assumed that the limits of normality are well defined by these values.

The lack of effect of insulin and adrenal cortical extract upon the enzyme preparation from the normal animal is indicated by the data in Table IV. The possible effect of these hormones upon the hemolysates from unwashed cells was also investigated, since there existed the possibility that the plasma

inhibiting factor might be pituitary in its origin. The fact that the plasma inhibiting effect is not released by the addition of insulin strongly suggests that this is not the case. Moreover, the inhibition by the plasma factor is not potentiated by addition of adrenal cortical extract.

Table V contains the data obtained from studies of diabetic rats. The animals were made diabetic by the intravenous injection of alloxan (40 mg. per kilo) after a 24 hour period of fasting. Thereafter they were allowed food and water *ad libitum*, except for the period 24 hours prior to cardiac puncture. Animals were not used until at least 5 days had elapsed

TABLE V

Hexokinase Activity of Hemolysates of Washed Erythrocytes from Hormone-Deficient Rats

All values are the average of duplicates and are expressed as c.mm. of CO_2 per hour. Experimental conditions the same as for Table III.

	Experiment No.	Blood glucose	No addition	With insulin*	With ACE†
		mg. per cent			
Diabetic	1	480	61	59	57
	2	330	84	90	84
	3	402	95	97	95
	4	350	95	94	93
	5	270	80	80	83
	6	340	108	99	99
	7	340	117	111	91
	8	320	85	87	92
Hypophysectomized	1	91	104		
	2	100	90	86	88
	3	100	104	90	95

* 100 γ per vessel; Lilly, crystalline.

† 0.1 ml. per vessel; Upjohn, alcoholic extract.

from the time of the injection of alloxan. Sugar was estimated on aliquots of the blood used for the enzyme preparations. It is apparent that the hexokinase activity of hemolysates from diabetic rats was not significantly different from that of the normal animals. Furthermore, the addition of insulin and adrenal cortical extract to these hemolysates also did not alter the hexokinase activity. Though data are not given, it was also found that unwashed red cells of the diabetic animal yielded hemolysates showing no essential difference from those obtained from unwashed normal cells.

The data obtained on hypophysectomized animals[1] are also given in Table V. The animals used showed the typical stigmata of hypophysec-

[1] We are indebted to Dr. Roy O. Greep for his kindness in performing the operation on these animals.

tomy, *i.e.*, long, silky hair, poor appetite, low activity, and atrophic adrenals on autopsy. Blood samples were not drawn until at least 2 weeks after the operation. The hexokinase activity of hemolysates prepared from their blood showed no significant variation from those obtained from normal animals. Addition of insulin or adrenal cortical extract was also without effect upon these hemolysates. Though data are not given, it was found that the plasma of hypophysectomized rats inhibited the hexokinase reaction to approximately the same extent as observed for normal plasma. This constitutes further proof that the plasma inhibiting factor is not pituitary in origin.

<center>DISCUSSION</center>

From the results presented, it is clear that we have not been able to demonstrate any effect of hormones from the pancreas, adrenals, or pituitary upon the activity of hexokinase obtained from the rat red blood cell. Wherein lies the cause of the difference between our observations and those of Cori *et al.* (3, 5, 14, 15), we cannot say. We feel reasonably certain that the method we have employed gives a true index to the activity of the hexokinase enzyme. Several possible explanations for the difference in results suggest themselves. First, the hexokinase of the red blood cell may be different from that found in muscle in that it is not under hormone control. In this connection, a comparison of the hexokinase activity of various tissues including malignant might be instructive. A second possibility is that the labile pituitary factor which Cori and coworkers found inhibitory to hexokinase had undergone destruction before our measurements were begun. We can only state that the lability of this pituitary factor has been uppermost in our minds throughout this work, and we have endeavored to work as rapidly as possible and at low temperatures in order to minimize such destruction. Indeed, the observation that washing red cells increased the hexokinase activity of the subsequent hemolysates suggested at first that the destruction of a labile pituitary factor was being encountered. However, the results we have presented do not seem to support this interpretation.

We do feel that it is important to emphasize that in a study of this sort one runs into a dilemma which certainly we have not resolved to our own satisfaction. We are concerned in this study with the relative rates of an enzymatic reaction. In setting up our enzyme test, we have followed the usual procedure of the enzyme chemist of choosing the conditions for the test which give the maximum rate of reaction. Is such a procedure justified in a study of the type we present here? Would it not be better to employ conditions which duplicate the level of activity found for the enzyme in the intact cell? If the answer is yes, as we are inclined to

believe, then the achievement of such conditions in a cell-free environment presents quite a problem. In the case of the rat red blood cell, this dilemma is pointed up by the following facts. Under the conditions of our test, the hemolysates from 1 cc. of red blood cells cause the disappearance of about 10 μM of glucose per hour. In rat whole blood, glucose utilization *in vitro* at 37.5° is approximately one-third of this value for a like number of red cells. It is obvious that in the study presented here the enzyme has been operating at a rate which is much nearer its maximum activity than is found within the intact red cell. It is thus possible that the only rate change to be observed under such conditions would be a diminution in rate. This being the case, it is the more unfortunate that the hormone reputed to effect the inhibition of hexokinase is also the least stabile of those under study.

We have more recently attempted to overcome the dilemma discussed above by studying the rate of glucose utilization by the intact rat red blood cell. In the experiments performed to date, we have duplicated most of the variation in hormone imbalance described here without, however, as yet achieving any alterations in the glucose utilization rate by the intact red blood cell.

Two factors which do affect the rate of hexokinase activity in hemolysates have been described here. These are a decrease in activity caused by (1) an increase in ionic strength, and (2) by the presence of plasma in the test system. It is thus obvious that this enzyme system is sensitive to minor changes in its environment which are apparently not related to hormone action and, therefore, such factors should be considered in any studies made on the hexokinase activity of other tissues.

SUMMARY

1. The hexokinase activity of hemolysates of rat red blood cells has been studied by a manometric technique. Chemical analyses for glucose and phosphorus compounds have shown that the following equation is satisfied during the reaction.

$$NaHCO_3 + glucose + ATP \rightarrow glucose\text{-}6\text{-}phosphate + ADP + CO_2$$

2. Mannose and fructose, but not galactose, can replace glucose in the reaction. The relative rates of phosphorylation of glucose, mannose, and fructose are in the ratio of 1.0:0.77:0.36.

3. The reaction is inhibited by plasma and it is necessary to wash red blood cells three times before hemolyzing in order to obtain maximum hexokinase activity. The plasma factor does not appear to be pituitary in origin. Increasing salt content of the reaction mixture also retards the reaction.

4. The activity of the enzyme as obtained from normal rats is not affected by the addition of insulin or adrenal cortical extract. Enzyme preparations from diabetic or hypophysectomized animals show no essential variation in activity from those of normal animals and are also uninfluenced by hormone additions.

BIBLIOGRAPHY

1. Berger, L., Slein, M. W , Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29**, 379 (1945–46).
2. Broh-Kahn, R. II., and Mirsky, I. A., *Science*, **106**, 148 (1947).
3. Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, **168**, 583 (1947).
4. Colowick, S. P., and Kalckar, II. M., *J. Biol. Chem.*, **148**, 117 (1943).
5. Cori, C. F., *Harvey Lectures*, **41**, 253 (1945–46).
6. Cori, G. T., and Slein, M. W., *Federation Proc.*, **6**, 245 (1947).
7. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
8. Kunitz, M., and McDonald, M. R., *J. Gen. Physiol.*, **29**, 393 (1945–46).
9. LePage, G. A., in Carter, H. E., Biochemical preparations, New York, **1**, 5 (1949).
10. Lohman, K., *Biochem Z.*, **233**, 460 (1931).
11. Meyerhof, O., *Biochem. Z.*, **246**, 249 (1932).
12. Meyerhof, O., and Geliazkowa, N., *Arch. Biochem.*, **12**, 405 (1947).
13. Nelson, N., *J. Biol. Chem.*, **153**, 375 (1944).
14. Price, W. II., Cori, C. F., and Colowick, S. P., *J. Biol. Chem.*, **160**, 633 (1945).
15. Price, W. II., Slein, M. W., Colowick, S. P., and Cori, G. T., *Federation Proc.*, **5**, 150 (1946).
16. Reid, E., Smith, R. II., and Young, F. G., *Biochem J.*, **42**, p. xix (1948).
17. Somogyi, M., *J. Biol. Chem.*, **160**, 69 (1945).
18. Speck, J. F., and Evans, E. A., Jr., *J. Biol. Chem.*, **159**, 71 (1945).
19. Stadie, W. C., and Haugaard, N., *J. Biol. Chem.*, **177**, 311 (1949).

THE FATE OF UTILIZED MOLECULAR OXYGEN AND THE SOURCE OF THE OXYGEN OF RESPIRATORY CARBON DIOXIDE, STUDIED WITH THE AID OF HEAVY OXYGEN*

By NATHAN LIFSON, GEORGE B. GORDON, M. B. VISSCHER, AND A. O. NIER

(*From the Departments of Physiology and Physics, University of Minnesota, Minneapolis*)

The present investigation was undertaken in an attempt to elucidate the fate of utilized molecular oxygen and to identify the source of the oxygen in respiratory carbon dioxide in the intact animal. As will be shown later, it is believed that erroneous conclusions were drawn from the only previous study with O^{18} as a tracer in connection with this problem.

Current concepts of the mode of oxidation in the animal body hold that the fate of molecular oxygen is to combine with hydrogen from substrates to form water.[1] Therefore, an animal breathing labeled oxygen should produce labeled water. The isotopic water thus formed would be added to the pool of already existing body water, increasing the isotopic concentration of the latter progressively with time.

The question of the source of oxygen in respiratory carbon dioxide may be conveniently discussed in terms of the conventional equation for the complete oxidation of glucose:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad (1)$$

Clearly, at least one-half of the oxygen in the carbon dioxide thus arising must come from some source other than the glucose itself. According to present concepts, carbon dioxide is released by decarboxylation and the extra oxygen in the carbon dioxide is previously introduced, not from molecular oxygen, but from body water. Hence glucose oxidation in the presence of labeled molecular oxygen and normal body water should form carbon dioxide entirely free of label; but glucose oxidation in the presence of normal molecular oxygen and isotopic water should yield

[1] No distinction is made here between the particular hydrogens removed from the substrate by dehydrogenation and those hydrogens which actually combine with oxygen to yield water, although in general they may be expected to be different. The former are presumed to add to the body water as hydrogen ions and the latter to be removed from the body water likewise as hydrogen ions.

carbon dioxide, the isotope concentration of which is at least 50 per cent that of the body water. Actually, glucose is thought to be metabolized to pyruvate by the glycolytic scheme of reactions, and pyruvate thereupon oxidized to carbon dioxide by the tricarboxylic acid cycle. In this case, it may well be that, before each of the predicted six decarboxylations per glucose molecule occurs, the oxygen of that released carbon dioxide will have been previously in more or less complete isotopic exchange equilibrium with, or derived from, body water. As a result, the isotopic concentration of the carbon dioxide would be raised toward equality with the isotope concentration of the body water. Similar considerations with differences in details would be expected to apply to the complete oxidation of fats and proteins.

Thus far the discussion has been of the source of the oxygen of respiratory carbon dioxide at the instant of its formation by internal respiration, presumably by decarboxylation. However, between the moment of its intracellular origin and its release from the animal in expired air, the carbon dioxide is provided with an opportunity to exchange oxygen with body water during each of the processes involved in transport and external respiration; *i.e.*, traversal of intracellular and interstitial fluid, carriage by the blood, entrance into the alveoli, and removal by ventilation.

At a pH less than 8, the predominant reaction is the following (1).

$$CO_2 + H_2O \underset{k_2}{\overset{k_1}{\rightleftharpoons}} H_2CO_2 \qquad (2)$$
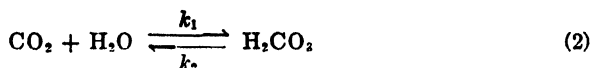
If either the carbon dioxide or the water initially contains all the labeled oxygen, by this reaction the isotope becomes distributed among all three reactants until the atom per cent O^{18} is the same in each, except as the process is influenced by fractionation effects. For the present purposes, the exchange equilibrium may be represented as follows:

$$CO^{16}O^{18} + H_2O^{16} \rightleftharpoons CO^{16}O^{16} + H_2O^{18} \qquad (3)$$
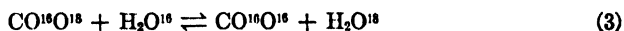
Employing a value of 0.10 sec.$^{-1}$ for the velocity constant, $k_1(H_2O)$, of Reaction 2, uncatalyzed, at 38° and pH 7.4, one can calculate from the appropriate equation of Mills and Urey (2) that 99 per cent of isotope equilibrium is reached in 138 seconds. However, the amounts of carbonic anhydrase in blood appear to be sufficient for exchange equilibrium to be achieved in a fraction of a second (3). Thus, even if the carbon dioxide arising intracellularly were not already in complete isotopic oxygen exchange equilibrium with water, such equilibrium might be expected to be attained subsequently, before exit from the body.

Consequently, both by current concepts of internal respiration and by the carbonic anhydrase-catalyzed exchange during transport and

external respiration, it would be anticipated (1) that the oxygen of respiratory carbon dioxide would approach equilibrium with body water, and (2) that utilized respiratory oxygen would soon find its way into body water.

The experiments to be reported appear to demonstrate that such is indeed the case. In the only previously published study of this question, a brief note by Day and Sheel (4), the inference was drawn that respiratory oxygen does enter directly into carbon oxidation and is exhaled as carbon dioxide, a conclusion at variance with predictions from current concepts of intermediary metabolism. It was therefore considered of importance to reinvestigate the question.

Methods

General Procedure—Two types of experiments were carried out: (a) that in which the atmosphere was enriched with O_2^{18} (O_2^{18} type)[2] and (b) that in which the body water was enriched with H_2O^{18} (H_2O^{18} type). In both types, the animal was placed in a closed metabolism system (see Fig. 1) filled with oxygen, and arranged for collection of dry respiratory carbon dioxide, for the sampling of the atmospheric gas, and for approximate determination of the rate of oxygen consumption. The collection periods, the number of which varied from two to four, were of 2 hours duration unless otherwise indicated. At the termination of the last collection period the animal was decapitated and a portion of body water obtained by vacuum distillation. Ordinarily, one atmospheric gas sample was taken at about the mid-point and one at the end of the experiment.

Experimental Details—Heavy molecular oxygen was concentrated by thermal diffusion. From the heavy molecular oxygen, heavy water was prepared by catalytic combination with normal hydrogen.

Strain A mice maintained on an unrestricted fox chow diet were employed. In the O_2^{18} type of experiment, the isotopic oxygen was added to the atmosphere of the system at I (see Fig. 1), after passing through a drying tube and carbon dioxide absorber. In the H_2O^{18} type of experiment body water was enriched by intraperitoneal injection of 1 ml. of H_2O^{18} of 8.47 atom per cent excess O^{18}, 2 to 3 hours before placing the mouse in the metabolism chamber. Preliminary trials had shown that this allowed more than enough time for the injected water to be absorbed.

The isotopic analyses for O^{18} were performed by means of the mass spectrometer. The atmospheric oxygen was analyzed directly from the

[2] The designation O_2^{18} is used to indicate molecular oxygen enriched with regard to O^{18}. It will be realized, however, that in the range of concentrations employed here most of the O^{18} is in $O^{16}O^{18}$ molecules and only a small proportion is in the $O^{18}O^{18}$ form.

sample bulb. In the case of respiratory carbon dioxide, the trap containing the solid sample, still immersed in liquid oxygen, was first evacuated, thus removing the gas phase. The trap was then removed from the refrigerant for introduction of volatilized carbon dioxide into the mass spectrometer. Since no special precautions were taken to mix the solid
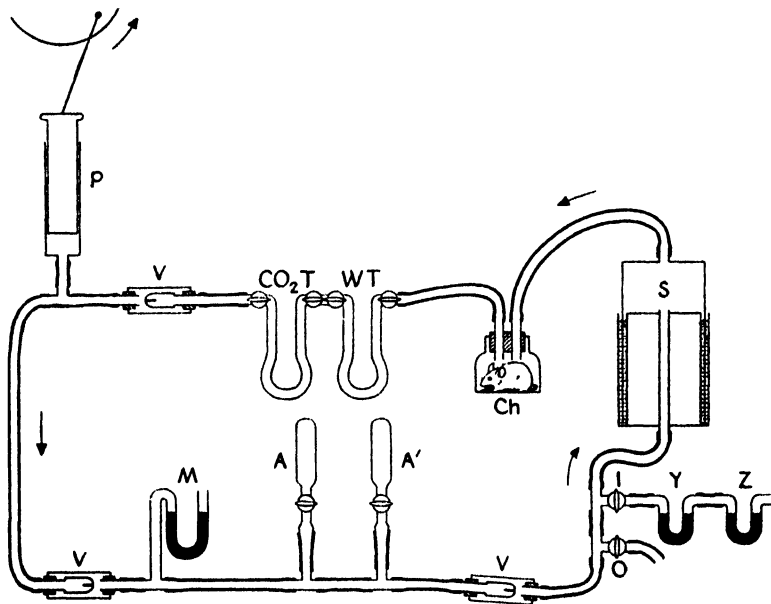
FIG. 1. *Ch*, animal chamber; *WT*, water trap (during the experiment immersed in alcohol-dry ice); CO_2T, carbon dioxide trap (during the experiment immersed in liquid oxygen or air); *V*, valve; *P*, syringe pump (sealed with mineral oil); *M*, mercury manometer; *A* and *A'*, evacuated sampling bulbs for atmospheric gas; *Y*, calcium chloride tube; *Z*, soda lime tube; *O, I*, stop-cocks; *S*, spirometer (sealed with mineral oil). Only one water trap and one carbon dioxide trap are shown; others were placed in parallel as necessary. Before each experiment, the system was flushed with oxygen introduced at *I* and removed through *O*, the segment between *I* and *O* being clamped during this procedure.

carbon dioxide, the sample of respiratory carbon dioxide actually analyzed for isotope can be considered only as some portion, but not at all necessarily a representative one, of the collection period in question. Body water was determined by a modification of the procedure of Cohn and Urey (5), in which the isotopic measurement is made on carbon dioxide previously equilibrated with the water sample to be analyzed. Carbonic anhydrase prepared by the method of Meldrum and Roughton (6) was added to the equilibrium chamber to hasten the attainment of isotopic

exchange equilibrium. All values are reported as atom per cent excess O^{18} referred to water as a standard; *i.e.*, the values for O_2^{18} and CO_2^{18} have been corrected for the fractionation which occurs between these molecules and H_2O. A fractionation factor of 1.039 was used for the equilibrium between water and carbon dioxide, a factor of 1.006 for the equilibrium between water and molecular oxygen (7). The over-all error of the isotopic analysis is of the order of 1 to 2 per cent of the determined isotopic concentrations.

Total body water was determined on five mice of the type employed by drying to constant weight at 110°. The mean value obtained was 62.6 per cent of the body weight, with a range of 61.0 to 65.7 per cent. From the rate of oxygen consumption, a predicted value for the isotope concentration in body water at the end of the O_2^{18} type of experiment was calculated as follows:

$$\text{Predicted } (O^{18})_{bw} = (O^{18})_a \times \cfrac{\cfrac{R \times 60 \times t \times 2}{22.4}}{\cfrac{0.626 \times \text{weight} \times 1000}{18} + \cfrac{R \times 60 \times t \times 2}{22.4}}$$

in which $(O^{18})_{bw}$ = the atom per cent excess O^{18} in the body water; $(O^{18})_a$ = the atom per cent excess O^{18} in the atmosphere; R = the rate of oxygen consumption in ml. per minute (corrected to standard pressure and temperature); t = the time of the experiment in hours; weight = the body weight of the mouse in gm.

In the H_2O^{18} type of experiments, a predicted value for the body water isotope level was computed from the dilution of the administered water by the preexisting body water, with a correction for formation of normal water by oxygen consumption.

In preliminary experiments it was found that mice exposed to O_2^{18} atmospheres under the conditions employed survived without obvious ill effects.

RESULTS AND DISCUSSION

The essential results, which are listed in Table I, are interpreted to indicate (1) that the oxygen of respiratory carbon dioxide is in exchange equilibrium with body water, and (2) that utilized molecular oxygen is converted to body water.

The following features of the results support the conclusion that the oxygen of respiratory CO_2 is in equilibrium with body water. (*a*) In one H_2O^{18} experiment (Table I, Experiment C) the isotope concentration in the respiratory carbon dioxide (0.51 to 0.52) is in each collection period practically identical with that observed for the body water (0.50). (*b*)

A value for the body water concentration in Experiment D is not at hand. It will be noted that in Experiment C the observed value for the body water agrees with that calculated from the dilution of the administered water. It would seem permissible to employ the corresponding calculated value for the O^{18} concentration for Experiment D. The value thus computed agrees well with that of O^{18} in the respiratory carbon dioxide collection periods. (c) In the O_2^{18} type of experiments (A and B, Table I), the O^{18}

TABLE I

Essential Experimental Results

The O^{18} values are in atom per cent excess. Mice were used in Experiments A to E; a rat in Experiment F.

Experiment	Type	Body weight	O^{18} in atmosphere	O^{18} in respiratory CO₂				O^{18} in body water		Remarks
				Period 1	Period 2	Period 3	Period 4	Observed	Predicted	
		gm.								
A	O_2^{18}	27.3	11.0 -10.9	0.08	0.15	0.38		0.43	0.43*	
B	"	29.0	5.00 - 4.97	0.00	0.02	0.13	0.21	0.21	0.20*	Collection Period 1, ¼ hr. before introduction of isotopic oxygen
C	H_2O^{18}	24.6	(0.00)†	0.51	0.52	0.51		0.50	0.51‡	1 ml. H₂O with 8.47 atom % excess O^{18} intraperitoneally
D	"	23.5	0.00	0.52	0.51				0.53‡	" "
E	Control		0.00 - 0.01	0.02	0.02			0.63§		Control; see text
F	O_2^{18}	66	3.98 - 3.94	0.01	0.04	0.06		0.06	0.06*	Collection periods 1 hr.

* Prediction from oxygen consumption; see methods; $R = 1.2$ ml. per minute for Experiment A; 1.3 ml. per minute for Experiment B.

† Assumed value; no analysis.

‡ Prediction from dilution of injected H_2O^{18} by body water.

§ Water introduced into respiration chamber

concentration in the last respiratory carbon dioxide sample may be compared with that of the body water. In Experiment A, the former is 0.38, the latter 0.43. In Experiment B, the value for the final carbon dioxide period is identical with that of the body water. It will be recalled that this comparison involves carbon dioxide collected for the 2 hour period preceding the time of the body water sample and that, moreover, the carbon dioxide analyzed was not necessarily a representative portion of the total collected sample. For these reasons, further detailed consideration of the comparison is not undertaken, except to point out that the isotope

concentrations in the final respiratory carbon dioxide and the body water are of the same order of magnitude.

The following features of the results support the conclusion that utilized molecular oxygen is converted to body water. (a) The O_2^{18} concentrations in the respiratory carbon dioxide in the O_2^{18} type of experiment (Table I, Experiments A and B) increased with time. Since the isotopic concentration of the carbon dioxide is found to reflect that of the body water, this observation is in qualitative agreement with the continued formation of isotopic water from the utilized oxygen. With approximately uniform oxygen consumption throughout the experiment, one might have anticipated a relatively uniform increase in the O^{18} concentration in the body water, and thus, in the respiratory carbon dioxide, from collection period to collection period. The failure to observe such a constant rise in Experiments A and B is attributed to the circumstance that the value for a given carbon dioxide sample is not necessarily representative of the collection period in question. (b) In the O_2^{18} type of experiments (A and B) there is good agreement between the observed body water values at the end of the experiment and those predicted from the rate of oxygen utilization. The significance of this finding is that the major portion of the utilized inspired oxygen is in fact accounted for in the body water.

Experiment E was carried out to estimate the degree to which the complete, or practically complete, isotopic equilibrium between body water and respiratory carbon dioxide may have occurred between the time the carbon dioxide left the animal body and its collection in the trap. Enriched water was added to the usual animal chamber, which was immersed in a water bath maintained at about 30°. Another chamber containing the mouse was placed between the spirometer and the chamber containing heavy water with a calcium chloride drying tube between the two chambers. In this manner, dry normal respiratory carbon dioxide from the mouse was provided with what may be taken as at least as good an opportunity to exchange with isotopic water before being trapped as occurred in the body water type of experiments. It was found that the O^{18} concentration of the respiratory carbon dioxide under these conditions was only 3 per cent of that of the isotopic water. Therefore, the complete equilibrium observed when the animal body water was enriched is attributed almost entirely to processes occurring within the animal body itself.

The O^{18} concentrations in the body water have a bearing on the size of the pool of oxygen in the body compounds other than water which is exchangeable with body water during the time of these experiments. Apparently this pool of oxygen was not great enough to be detected in the form of a discrepancy between observed body water isotopic concen-

trations and values computed either (a) in the O_2^{18} type of experiment by the oxygen consumption method or (b) in the H_2O^{18} type of experiment from the extent of the anticipated dilution of injected water. Any exchangeable oxygen would have operated to reduce the observed value below the calculated one, an effect which was not actually demonstrable.

Day and Sheel (4), using rats and an artificial atmosphere containing oxygen equivalent in isotope composition to water of 300 parts per million excess density (0.24 atom per cent excess O^{18}), found that the expired carbon dioxide contained oxygen isotopes in proportions corresponding to about 40 parts per million excess density (0.032 atom per cent excess O^{18}); i.e., 13 per cent of the concentration of the inspired oxygen. As previously noted, these investigators interpret their findings as evidence that respiratory oxygen enters directly into carbon oxidation and is exhaled as carbon dioxide.

The results of the present experiments are in apparent disagreement with those of Day and Sheel in that the former provide no evidence for direct combination of carbon with molecular oxygen to yield respiratory carbon dioxide; all the O^{18} in respiratory carbon dioxide can be explained by equilibration with body water. To investigate whether a species difference between the rat and mouse may account for the discrepancy between the two sets of data, an experiment was carried out in which a rat was placed in an atmosphere enriched with heavy oxygen. The findings (Table I, Experiment F) exhibit relationships similar to those for the corresponding mouse experiments. The isotopic concentration in the carbon dioxide increased with time, but during the 3rd hour its value was still only about 1.5 per cent that of the utilized oxygen. Moreover, this value was identical with that of the body water at the end of the experiment and with the value predicted from the rate of oxygen consumption and the amount of body water. Since the duration of the experiment of Day and Sheel is not given, conceivably these investigators exposed the rat to the heavy oxygen atmosphere for much longer periods than those employed in the present studies. In this case, enough oxygen may have been converted to body water to enrich it to the extent indicated by the respiratory carbon dioxide values reported in their paper.

Although we have not in these experiments assessed the degree to which the isotopic exchange equilibrium between respiratory carbon dioxide and body water is established between the time of intracellular origin of the carbon dioxide and its exit from the animal, there is reason to believe it to be complete. Without such an evaluation no inferences are permissible from the present results concerning the isotopic composition of the respiratory carbon dioxide at the moment of formation in internal respiration, by decarboxylation or otherwise.

SUMMARY

With the aid of heavy oxygen, it has been found in the mouse and rat that (1) the oxygen in respiratory carbon dioxide is in isotopic equilibrium with the oxygen of body water, and that (2) at least a large majority, and perhaps all, of utilized molecular oxygen is soon converted to body water.

BIBLIOGRAPHY

1. Faurholt, C., *J. chim. phys.*, **21**, 400 (1924).
2. Mills, G. A., and Urey, H. C., *J. Am. Chem. Soc.*, **62**, 1019 (1940).
3. Roughton, F. J. W., *Physiol. Rev.*, **15**, 241 (1935).
4. Day, J. N. E., and Sheel, J. P., *Nature*, **142**, 917 (1938).
5. Cohn, M., and Urey, H. C., *J. Am. Chem. Soc.*, **60**, 679 (1938).
6. Meldrum, N. U., and Roughton, F. J. W., *J. Physiol.*, **80**, 113 (1933–34).
7. Dole, M., *Science*, **109**, 77 (1949).

BIOCHEMICAL PROPERTIES OF SUCCINOXIDASE FROM SALMONELLA AERTRYCKE*

By ERNEST KUN† AND LEO G. ABOOD

(From the Departments of Pharmacology and Medicine, University of Chicago, Chicago)

In the course of studies of the biochemical nature of the endotoxin of *Salmonella aertrycke* (1–3), it was found that the addition of succinate to the endotoxin caused a marked oxygen uptake. This oxidation occurred even four to seven months after the preparation of the endotoxin when the bacterial material was kept under sterile conditions and stored in the cold. Repeated tests for sterility by culturing[1] revealed that no living microorganism was present in the endotoxin which had been sterilized by heating it three times for 25 minutes at 60°. It appeared, therefore, of interest to investigate the succinoxidase properties of this bacterial material, since the previous findings suggested its enzymatic nature.

Materials and Methods

Preparation and Properties of Enzyme—The original material, referred to as *Salmonella* endotoxin, was prepared in Dr. C. Phillip Miller's laboratory, by a procedure described elsewhere (1). This endotoxin could be separated by centrifugation at 3500 × *g* for 60 minutes at 0° into a light insoluble sediment and a supernatant, containing the soluble material of the microorganisms. After the supernatant was decanted, the sediment was washed, and finally resuspended in 0.1 M phosphate buffer at pH 7.4. This suspension was lyophilized. The resulting product was a light powder which was stored at 0°. This preparation formed a homogenous suspension in distilled water or phosphate buffer and could be removed by centrifugation at 3500 × *g* in 60 minutes. Nitrogen and phosphorus analyses revealed the following results, expressed in terms of micrograms per mg. of dry enzyme (lyophilized weight): total N 41.2, phospholipide P 3.92, nucleic acid P 1.35, phosphoprotein P 8.45. Phosphorus analyses were carried out by the method of Schneider (4). The enzyme protein con-

† Present address, Division of Infectious Disease, Tulane University School of Medicine, New Orleans.

[1] The sterility tests were kindly carried out by Miss M. A. Bohnhoff in the Department of Medicine.

tained oxidizable sulfhydryl groups (0.374 γ per mg. of dry enzyme), as determined by the method of Anson (5).

This enzyme preparation could oxidize succinate aerobically, as measured in the Warburg respirometer, or anaerobically, as determined by the decolorization of methylene blue or by the tetrazolium method (6). The optimum pH of the enzyme activity was found to be 7.4 (Fig. 1). The enzyme activity was rapidly destroyed by alkaline (above pH 8) medium.
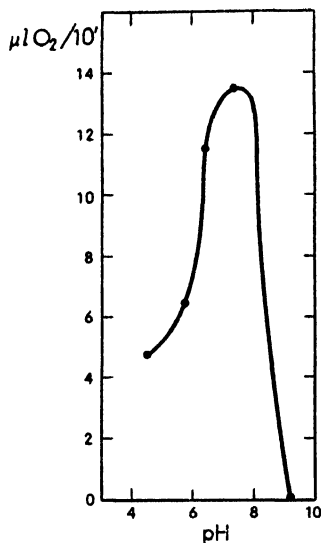
FIG. 1. Effect of pH on *Salmonella* succinoxidase. Each Warburg flask contained 5 mg. of lyophilized enzyme, 0.5 ml. of 0.1 M sodium succinate, and phosphate buffer (pH 4.2 to 11.2). The gas phase was air; temperature 37°.

Oxidation of Succinate—The enzymatic activity of this lyophilized preparation was tested with succinate, fumarate, malate, acetate, citrate, pyruvate (in presence of adenosine triphosphate), and glucose as substrates. No measurable oxygen uptake occurred except with succinate. In the presence of 0.5 ml. of 0.1 M sodium succinate the rate of oxygen consumption was directly proportional to time and the amount of enzyme in the course of an experimental period of 60 minutes (Fig. 2). The presence of inorganic phosphorus was not necessary for the oxidation of succinate. After thorough washing with distilled water and subsequent centrifugations (until the supernatant did not contain any inorganic phosphorus) the enzyme could oxidize succinate in phosphate-free trishydroxylmethylaminoethane buffer of pH 7.4 (7) to the same extent as in phosphate buffer. Calcium ion did not influence the rate of succinate oxidation.

Since it is generally accepted that in animal succinoxidase the activator of molecular oxygen is the cytochrome system, it was of interest to establish whether or not the *Salmonella* enzyme contained this system. Hydroquinone (8, 9) and ascorbate (10) were used as secondary substrates for the assay of cytochrome oxidase. Heating in a boiling water bath for 10 minutes completely destroyed the succinoxidase activity of the preparations. Heated samples were, therefore, used as controls for the cytochrome
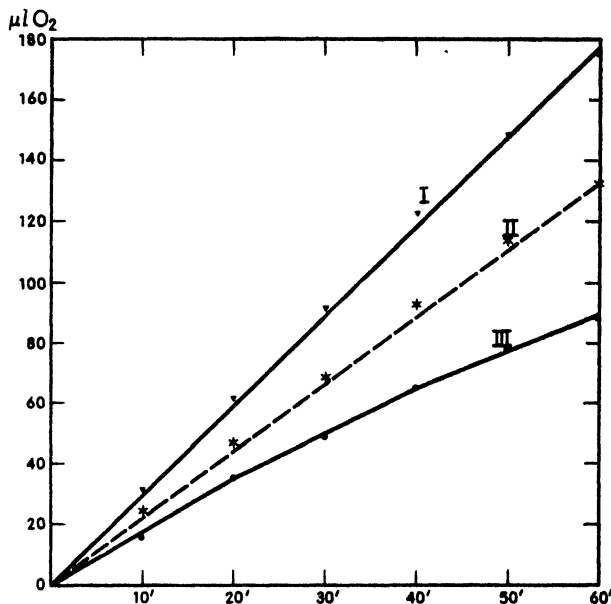
FIG. 2. Oxidation of succinate by dried *Salmonella* preparation. Each flask contained 5 mg. of lyophilized enzyme, 0.5 ml. of 0.1 M sodium succinate, and phosphate buffer (pH 7.4). Curve I, no inhibitor; Curve II, + 1.5 mg. of cytochrome *c;* Curve III, + 50 γ of methylene blue. The abscissa represents time in minutes.

oxidase assay. After the addition of cytochrome and secondary substrates (ascorbate, hydroquinone) no difference was observed between the oxygen consumption of the unheated and heat-inactivated samples, suggesting the absence of cytochrome oxidase. The use of heat-inactivated blanks in the cytochrome oxidase assay was of importance, since considerable nonenzymatic oxygen uptake occurred when cytochrome, ascorbate, or hydroquinone and heat-inactivated enzyme preparations were shaken at 37° in a respirometer. The importance of the use of heated controls has been emphasized by Stotz *et al.* (8, 9). The effect of the *Salmonella* enzyme on cytochrome *c* was also measured spectroscopically. It was found that

after the addition of the enzyme to a solution of cytochrome *c* the characteristic absorption band of reduced cytochrome appeared at 550 mμ. This reduction of cytochrome *c*, which also occurred anaerobically, was not affected by the addition of succinate. The reduced cytochrome *c* was not reoxidized by the *Salmonella* enzyme.

The effect of cytochrome *c* on the anaerobic and aerobic oxidation of succinate by the enzyme preparation was also examined. The rate of anaerobic reduction of methylene blue in the presence of succinate was not affected by the addition of cytochrome *c*, while the aerobic oxidation of succinate was inhibited (Fig. 2). The type of inhibition was determined by the use of the Lineweaver-Burk modification of the Michaelis-Menten equation (11). In a series of Warburg respirometers varying amounts (0.05 to 2.0 ml.) of 0.1 M sodium succinate and a constant amount of *Salmonella* enzyme (5 mg.) were suspended in phosphate buffer (pH 7.4) and oxygen uptake was measured at 37° for 60 minutes.

By the method of Lineweaver and Burk, K_s was found to be 2.2 $\times$ 10^{-3} M succinate. In order to determine the type of inhibition caused by cytochrome *c*, a second series of respirometers was set up, containing the same amount of enzyme and substrate as in the first series, but in addition a constant amount (2.5 mg. per flask) of cytochrome *c*. Since the intersection of K_I/V_m (Fig. 3) and the ordinate was found to be the same as in the case of the uninhibited system (K_s/V_m), *i.e.* the velocity of the reaction at infinite substrate concentrations was identical in the presence and absence of the inhibitor, the type of inhibition was, according to Ebersole *et al.* (12), a competitive one (Fig. 3). The observation that the aerobic oxidation of succinate was inhibited by cytochrome and the anaerobic dehydrogenation remained unaffected suggested that the aerobic part of the *Salmonella* enzyme was involved in the inhibition of succinate oxidation by cytochrome *c*.

The effect of temperature on the *Salmonella* enzyme was determined in the Warburg apparatus. Each flask contained 10 mg. of enzyme and 0.5 ml. of 0.1 M sodium succinate in phosphate buffer at pH 7.4. After temperature equilibration three subsequent 10 minute readings were taken at 32.2°, 37°, 38.6°, and 41.8°. Since the reaction of succinate and *Salmonella* enzyme was linear under given experimental conditions during the first 60 minutes (see Fig. 2), K was defined as the moles of succinate oxidized per 10 minutes per 10 mg. of enzyme. The amount of oxidized succinate was computed on the basis that 1 mole of oxygen was used for the oxidation of 1 mole of succinate (see below). According to the van't Hoff equation when ln K was plotted against the reciprocal of the absolute temperature $(1/T)$, a straight line $(-E/R)$ was obtained. From the slope of this straight line E (the heat of activation) was calculated to be 10,470 calories per mole of succinate (Fig. 4).
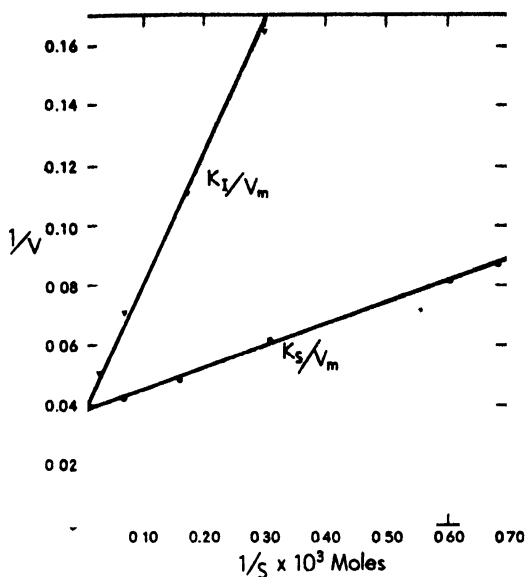
FIG. 3. Competitive inhibition of the aerobic oxidation of succinate by *Salmonella* succinoxidase in the presence of cytochrome c. V = velocity of reaction in Q_{O_2}, S = succinate concentration, $K_s = ((V_m/V) - 1)S$, when $V_m = 2V$, $K_s = S$, V_m = maximum velocity, and $K_I = K_s$ in the presence of cytochrome c.
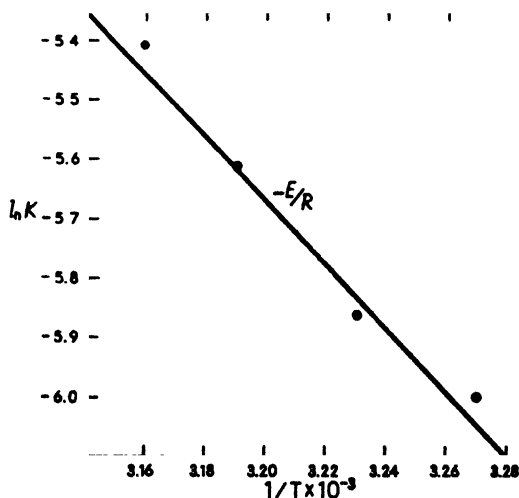
FIG. 4. Effect of temperature on the reaction velocity of *Salmonella* succinic dehydrogenase. $-E/R = (\Delta \ln K)/(\Delta l/T)$; K = moles of succinate, oxidized by 10 mg. of lyophilized enzyme in 10 minutes; T = absolute temperature.

In order to study further properties of the *Salmonella* enzyme, a number of enzyme inhibitors were tested, the results of which are summarized in Table I. While malonate and iodoacetamide in a final concentration of 0.01 M completely inhibited succinate oxidation, cyanide, azide, and selenite were considerably less effective. It is of interest that penicillin impurity, an intermediate product in the commercial production of penicillin, which was found to protect mice against endotoxins of Gram-negative organisms (13), inhibited this bacterial enzyme. Crystalline penicillins G and K were virtually ineffective, which was analogous to a previous finding made on the glycolysis of mouse tissues (14).

TABLE I

Inhibition of Salmonella Succinoxidase

Inhibitor	Q_{O_2}	Per cent inhibition
Control, no inhibitor .	27.2	
0.01 M sodium cyanide	9.9	63.5
0.01 " " azide	23.0	15.8
0.01 " " selenite	20.6	22.5
1 mg. penicillin impurity	6.1	78.0
10,000 units crystalline penicillin	25.6	7.0
0.01 M iodoacetamide	0	100
0.01 " malonate	0	100
1 mg. triphenyltetrazolium chloride	16.4	43.0
30 γ methylene blue ..	17.5	36.0

Each Warburg flask contained 2.5 mg. of *Salmonella* enzyme, 0.5 ml. of 0.1 M sodium succinate, and the inhibitor in a final volume of 3 ml. The oxygen uptake was measured for 60 minutes at 37°.

Direct spectroscopic measurement as well as cytochrome oxidase assays did not demonstrate the oxidation of cytochrome c by the *Salmonella* succinoxidase; therefore, it was necessary to postulate some other system which could serve as an oxygen activator. Since flavoproteins were known as direct acceptors of molecular oxygen, attempts were made to detect a flavin constituent of the *Salmonella* enzyme. A sample of 300 mg. of the *Salmonella* enzyme was extracted with acid acetone according to the procedure of Scott *et al.* (15). The absorption spectrum of the clear, pale yellow extract was determined in the Beckman spectrophotometer. Absorption maxima for the acid acetone extract were obtained at 375 to 390 mμ and at 445 to 450 mμ (Fig. 5). The addition of sodium hydrosulfite to the neutralized enzyme extract resulted in decolorization. The absorption spectrum of the acid acetone extract was compared with that of riboflavin (4.8 γ per ml.) dissolved in acid acetone. Maxima for ribo-

flavin were obtained at 375 mμ and at 445 to 450 mμ, values which corresponded with those found in the literature (16). On the basis of these spectrophotometric measurements, it cannot be stated with certainty that the enzyme extract contains flavin; however, the correspondence of maxima at 375 mμ and at 445 to 450 mμ with those of riboflavin might suggest the presence of a flavin-like compound. Attempts to isolate a soluble flavoprotein from the enzyme were unsuccessful. Assuming that a flavin-like catalyst is involved in the oxidation of succinate by the *Salmonella* enzyme, the formation of hydrogen peroxide would be expected. It was found that the *Salmonella* enzyme contained catalase activity, 1 mg.
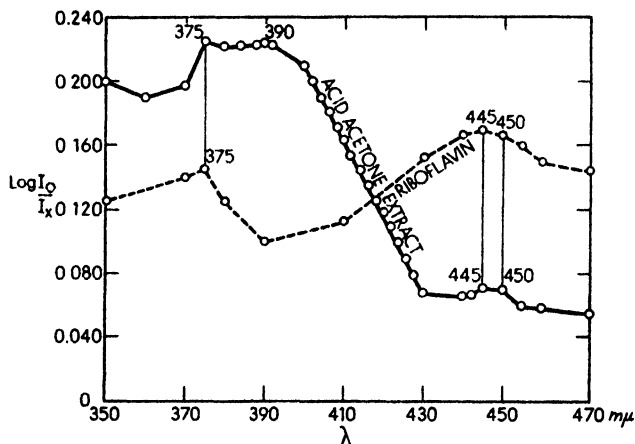
FIG. 5. Absorption spectrum of the acid-acetone extract of the *Salmonella* succinoxidase. Measurements were made on the Beckmann DU spectrophotometer. Acid acetone (15) was used as blank. The absorption spectrum of the extract was compared with that of riboflavin (4.8 γ per ml.), which was dissolved in acid acetone. Maxima were obtained for riboflavin at 375 mμ and 445 to 450 mμ and for the extract at 375 to 390 mμ and at 445 to 450 mμ.

of enzyme splitting 520 γ of hydrogen peroxide per 5 minutes at room temperature. Pyrogallol could be used as an oxygen acceptor in the presence of hydrogen peroxide and enzyme, and thereby peroxidase activity could be also demonstrated. The formation of hydrogen peroxide and the action of peroxidase during succinate oxidation could be shown in a system containing enzyme, succinate, and pyrogallol. If no succinate was added, a very slow darkening of the mixture was observed (probably due to traces of hydrogen peroxide), while the reaction mixture turned dark in 10 to 20 minutes in the presence of succinate. The mechanism of the cyanide inhibition of the aerobic oxidation of succinate by the *Salmonella* enzyme might be explained on the basis that cyanide inhibited the destruction

of H_2O_2. Under anaerobic conditions, when methylene blue or tetrazolium was present as hydrogen acceptor, cyanide did not influence the rate of reduction of the dye.

Succinate is oxidized to fumarate by the cytochrome-linked succinoxidase system according to the following equation.

$$1 \text{ mole succinate} + 0.5 \text{ mole } O_2 = 1 \text{ mole fumarate} + H_2O$$

During the complete oxidation of succinate by the *Salmonella* preparation, the amount of oxygen consumed per mole of succinate was dependent on the amount of enzyme present in the reaction mixture. With 2 to 2.5 mg. of enzyme per flask 1.1 to 1.3 $\times$ 0.5 mole of oxygen was utilized per mole of succinate. If, however, 5 to 10 mg. of enzyme were used, the ratio, 1 mole of oxygen per mole of succinate, was approached. In three Warburg vessels, each containing 10 mg. of enzyme and 1 ml. of 0.1 M succinate, oxygen consumption and fumarate formation were determined. Assuming that 1 mole of oxygen per mole of succinate was absorbed, the amount of fumarate formed in 1 hour was calculated to be 3.26 mg. per flask. The contents of the Warburg flasks were analyzed for fumarate by permanganate titration and 3.0 to 3.1 mg. of fumarate per flask were recovered. It is possible that in the presence of sufficient oxidizable material (10 mg. of enzyme), the 1:1 oxygen-succinate molar ratio was due to the peroxidase activity of the preparation, while with smaller amounts of enzyme catalase activity prevailed.

Attempts to demonstrate the reverse of the reaction succinate $\rightleftharpoons$ fumarate by the *Salmonella* preparation showed that the enzyme had a fumaric hydrogenase activity. This was demonstrated in a system similar to that reported by Fischer and Eysenbach (17). Each Thunberg tube contained 10 mg. of enzyme suspended in 1 ml. of 0.1 M phosphate buffer of pH 7.4, 1 ml. of 2×10^{-4} M methylene violet, 2 ml. of distilled water, and 1 ml. of 0.2 M sodium fumarate in the side arm. The air was removed from the tube by passing a gas mixture of 95 per cent nitrogen and 5 per cent CO_2 through the reaction mixture for 2 minutes. The methylene violet was then reduced by the addition of 0.2 ml. of freshly prepared 0.5 per cent sodium hydrosulfite. The tube was immediately evacuated and the time of restoration of color measured at room temperature. Control tubes contained 1 ml. of distilled water in the side arm instead of fumarate. It was found that no color occurred in the blank even after 48 hours of incubation, while in the presence of fumarate the color of the methylene violet was completely restored at room temperature in 15 to 25 minutes. Since it was reported (18) that succinic dehydrogenase in the absence of air in the presence of enzymatically reduced coenzyme I can reduce fumarate to succinate, we further investigated the question whether or not the

succinic dehydrogenase of the bacterial preparation was responsible for the fumaric hydrogenase activity. It was found that the anaerobic restoration of color of reduced methylene violet occurred undisturbed in the presence of 0.02 M malonate or iodoacetamide. These inhibitors completely blocked succinic dehydrogenase activity. From this observation it could be ascertained that the succinic dehydrogenase and fumaric hydrogenase activity of the enzyme preparation could not be due to the same component of the system.

DISCUSSION

A simple procedure for the preparation of a stable succinoxidase from *Salmonella aertrycke* has been described. Experiments with this enzyme demonstrated that it could oxidize succinate by molecular oxygen. Attempts to demonstrate cytochrome system in the preparation were unsuccessful. Since the acid acetone extract of the *Salmonella* enzyme had a flavin-like spectrum, and since evidence was obtained for the formation of hydrogen peroxide during aerobic succinate oxidation, it appeared reasonable to assume that the reaction of molecular oxygen was mediated by a catalyst similar to flavoprotein. The existence of two distinct factors in the *Salmonella* succinoxidase, namely the succinic dehydrogenase and another factor capable of reducing fumarate in presence of reduced methylene violet, appeared to support this assumption. It is interesting to mention in connection with this explanation that yeast fumaric hydrogenase was found by Warburg and Christian to be a flavoprotein (19).

Flavoprotein has been postulated to be a part of the animal succinoxidase system (20). Keilin and Hartree (21) found some evidence by means of spectroscopic analysis which indicated the participation of a flavin component in aerobic succinate oxidation. Lehmann and Märtensen (22) observed the formation of hydrogen peroxide during aerobic succinate oxidation, suggesting an incomplete oxidation of reduced flavin by the cytochrome system. Further experiments to demonstrate the rôle of flavin in succinate oxidation were carried out by Axelrod, Potter, and Elvehjem (23) and Axelrod, Swingle, and Elvehjem (24) who found that in riboflavin-deficient rats a component of the succinoxidase system was diminished. This component was not succinic dehydrogenase or the cytochrome system, and could be restored by riboflavin feeding. Since other flavin enzymes, such as xanthine and D-amino acid oxidase, also decreased in riboflavin-deficient animals (25, 26), these observations seemed to corroborate the assumption that flavin might be a part of succinoxidase. These observations, however, based on circumstantial evidence, appear to support the conclusions drawn from the studies on the *Salmonella* enzyme.

The inhibition of aerobic succinate oxidation by cytochrome c is of

special interest. Kinetic studies revealed a competitive type of inhibition of the aerobic succinate oxidation by cytochrome c. The competition occurred between cytochrome c and a part of the succinoxidase which participates in aerobic oxidation. Cytochrome c was reduced by the *Salmonella* enzyme but was not reoxidized. Similar reduction of cytochrome c by animal succinic dehydrogenase, which was not affected by the addition of succinate, was reported by Hogeboom (27). It is possible that the reduction of cytochrome c is brought about by the same component (flavin) which activates molecular oxygen in the *Salmonella* preparation and this circumstance could explain the competitive inhibition of succinate oxidation by cytochrome c.

SUMMARY

1. Succinoxidase was prepared from *Salmonella aertrycke* by lyophilization of the insoluble material of the sterile bacterial endotoxin.

2. Some properties of the enzyme (N, P, and SH analyses, pH optimum, kinetics of succinate oxidation) were determined.

3. The enzyme did not contain cytochrome oxidase, but could reduce cytochrome c.

4. Cytochrome c inhibited the aerobic but not the anaerobic oxidation of succinate. The inhibition was of competitive type. The most potent inhibitors of the *Salmonella* enzyme were iodoacetamide and malonate.

5. The preparation had catalase and peroxidase activity.

6. In the presence of reduced methylene violet a component of the enzyme which was not inhibited by malonate or iodoacetamide could reduce fumarate to succinate.

BIBLIOGRAPHY

1. Kun, E., *Proc. Soc. Exp. Biol. and Med.*, **66**, 197 (1947).
2. Kun, E., and Miller, C. P., *Proc. Soc. Exp. Biol. and Med.*, **67**, 221 (1948).
3. Kun, E., *Proc. Soc. Exp. Biol. and Med.*, **68**, 496 (1948).
4. Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).
5. Anson, M. L., *J. Gen. Physiol.*, **24**, 399 (1941).
6. Kun, E., and Abood, L. G., *Science*, **109**, 144 (1949).
7. Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, **62**, 33 (1946).
8. Stotz, E., Sidwell, A. E., Jr., and Hogness, T. R., *J. Biol. Chem.*, **124**, 733 (1938).
9. Stotz, E., Altschul, A. M., and Hogness, T. R., *J. Biol. Chem.*, **124**, 745 (1939).
10. Potter, V. R., in Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 95 (1946).

11. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56,** 658 (1934).
12. Ebersole, E. R., Guttentag, C., and Wilson, P. W., *Arch. Biochem.*, **3,** 399 (1943–44).
13. Miller, C. P., Hawk, W. D., and Boor, A. K., *Science*, **107,** 118 (1948).
14. Kun, E., *Science*, **108,** 117 (1948).
15. Scott, M. L., Hill, F. W., Norris, L. C., and Heuser, G. F., *J. Biol. Chem.*, **165,** 65 (1946).
16. Warburg, O., and Christian, W., *Biochem. Z.*, **298,** 150 (1938).
17. Fischer, F. G., and Eysenbach, H., *Ann. Chem.*, **530,** 99 (1937).
18. Green, D. E., and Dewan, J. G., *Biochem. J.*, **31,** 1069 (1937).
19. Warburg, O., and Christian, W., *Biochem. Z.*, **298,** 368 (1938).
20. Barron, E. S. G., *Physiol. Rev.*, **19,** 184 (1939).
21. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **129,** 277 (1940).
22. Lehmann, J., and Märtensen, E., *Skand. Arch. Physiol.*, **75,** 61 (1937).
23. Axelrod, A. E., Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **142,** 85 (1942).
24. Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *J. Biol. Chem.*, **145,** 297 (1942).
25. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **140,** 725 (1941).
26. Axelrod, A. E., Sober, H. A., and Elvehjem, C. A., *J. Biol. Chem.*, **134,** 749 (1940).
27. Hogeboom, G. H., *J. Biol. Chem.*, **162,** 739 (1946).

PURIFICATION OF THE HYPERGLYCEMIC-GLYCOGENOLYTIC FACTOR FROM INSULIN AND FROM GASTRIC MUCOSA*

By EARL W. SUTHERLAND, CARL F. CORI, ROBERT HAYNES,
AND NORMAN S. OLSEN

(*From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis*)

The occurrence of a hyperglycemic-glycogenolytic (H-G) factor in certain commercial insulin preparations (1) led to a search for this factor in the pancreas and in other tissues. Apart from the pancreas and the mucosa of certain portions of the gastrointestinal tract, all other tissues were found to be free of the factor. The method of extraction and precipitation was similar to that used for the isolation of insulin. In the present paper the properties of the factor from pancreas and from the gastric mucosa of the dog are compared at different stages of purification.

The quantitative assay of the factor is based on its property of stimulating glycogenolysis in isolated liver slices. Insulin, when present, does not interfere in this assay. A description of the method has been previously given and it is somewhat amplified in this paper. The hyperglycemic response resulting from injection into intact animals has also been determined. Starting with an amorphous insulin powder as source of the pancreatic factor, fractions have been obtained which were 10 times as active in the liver test system as the original material. These preparations when injected intravenously into rabbits produced a hyperglycemia of about 40 minutes duration without subsequent hypoglycemia; they were therefore free of insulin. The factor extracted from the gastric mucosa of the dog showed properties which were essentially the same as those of the pancreatic factor. On injection into rabbits the blood sugar curves showed the same characteristics as those observed after injection of the pancreatic factor.

Methods

Liver slices were prepared and incubated as described previously (1, 2). For assay purposes it is desirable that slices of uniform thickness be used, since thick slices (*e.g.* 0.8 to 1.0 mm.) tend to produce less glucose per unit weight than slices of medium thickness (*e.g.* 0.4 to 0.5 mm.). Best results are obtained when slices are matched within 15 per cent with respect to weight per unit area and when each assay is carried out in duplicate. If

the liver is kept well chilled, slices may be prepared from it over a period of 40 minutes; this permits the preparation of a large number of slices from a single animal, especially if one person prepares the slices while another weighs them.

Examples will be given for comparative assays of the same preparation of H-G factor on liver slices of different animals. The agreement was satisfactory with the exception of two cases in which the variation was 25 to 30 per cent. For each individual assay it is desirable to use slices from adjacent areas of the liver.

Since pH and salt content of the incubating fluid have some effect on the glucose output from slices, solutions to be tested were dialyzed in advance against the chloride-phosphate buffer, or suitably diluted and adjusted with respect to pH. Unless stated as an approximate value, all pH measurements were carried out with a glass electrode.

Analytical methods have been reported previously except for the use of a micro protein method (3) which has been modified for the present study and which proved to be of great value when fractionating small amounts of protein.[1] To 0.5 ml. of protein solution there are first added 5.0 ml. of a dilute alkaline copper solution (prepared twice weekly by adding 1 ml. of 2 per cent $CuSO_4 \cdot 5H_2O$ and 1 ml. of 4 per cent sodium tartrate to 100 ml. of a 4 per cent Na_2CO_3 solution). The mixture is incubated 10 to 15 minutes at 45°, at which time 0.5 ml. of phenol reagent (Folin-Ciocalteau) diluted 1:3 with water is added. Each tube is mixed by inversion immediately after addition of the phenol reagent. The tubes are then allowed to stand 15 minutes at room temperature before being read in the Klett-Summerson photoelectric colorimeter with Filter 660. The amounts of protein determined were usually between 0.025 and 0.25 mg., although smaller amounts can be measured. Two or more concentrations of insulin were used as protein standards. The results agreed closely with those obtained with the biuret method of Weichselbaum (4).

The per cent of maximal stimulation was calculated as described previously (1). In order to compare the activity of different preparations, it was desirable to determine the effect of three or more concentrations. As shown in Fig. 1 the relation between the amount of factor added and the response is approximately linear up to 70 per cent of the maximal response. From such plots it was possible to determine the amount of protein required to give half maximal stimulation of glucose output from liver slices, and most of the results are expressed in these terms. The insulin used in the present investigation was supplied by Eli Lilly and Company and was of two types: an amorphous insulin preparation with 20 to 21 units per mg.,

and a crystalline zinc preparation with 25 units per mg. The amorphous insulin was used except when stated otherwise.

Fractionation of Insulin

Assay of Insulin for H-G Factor—The insulin preparations used for fractionation were first assayed for their content of H-G factor (Fig. 1). It was found that the amorphous preparation, as well as the crystalline zinc insulin preparation used, gave half maximal stimulation when added in amounts of approximately 6.5 γ.[2] Another sample of crystalline zinc insulin contained considerably less factor per unit weight, while insulin preparations obtained from the Novo Laboratories were essentially free of
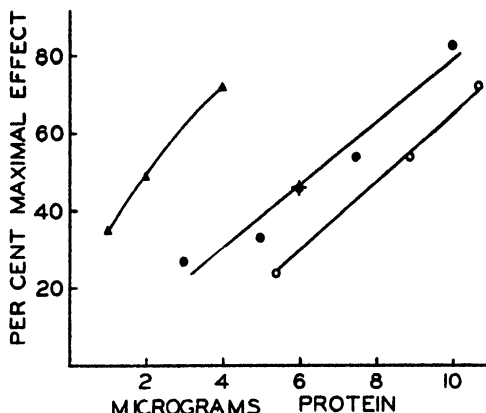
Fig. 1. Assay curves for H-G factor. ●, amorphous insulin, ✖ representing assay on another animal. O, amorphous insulin after incubation for 2.5 hours in 0.08 N KOH at 37°. △, fibril filtrate of amorphous insulin.

the factor (2). A recent preparation of crystalline zinc insulin obtained from Eli Lilly and Company was also free of the H-G factor.

Presence of H-G Factor in Insulin Fibril Filtrates—The formation of fibrils from insulin has been described by Waugh (5). These fibrils can be removed by filtration and it seemed possible that the H-G factor might be found in the filtrate. Fibrils were formed by Waugh's method from amorphous or crystalline zinc insulin and filtered off on No. 50 Whatman paper with suction. While only 10 per cent of the original H-G activity appeared in the filtrate, the activity per unit of protein was increased 3 to 4 times by this procedure (Fig. 1). It was shown that the remainder was not destroyed by the treatment but was trapped in the fibril mat. Regeneration of the insulin and repeated fibril formation resulted in the

[2] The total volume of fluid in the liver test system was 1.2 ml. in each case.

appearance of more H-G factor in the fibril filtrate, but in general these filtrates were less active per unit of protein than the first fibril filtrate.

Some attempts were made to purify the factor in the fibril filtrates by isoelectric precipitation at pH 6 in the presence of zinc ions. After two such precipitations, and removal of inactive material which became insoluble at pH 8, a fraction was obtained which showed half maximal activity at 1.0 γ of protein per test (Fig. 2). Microscopically this fraction consisted of uniform spheroids; it still contained small amounts of insulin, as shown in Fig. 3. A third isoelectric precipitation yielded material which
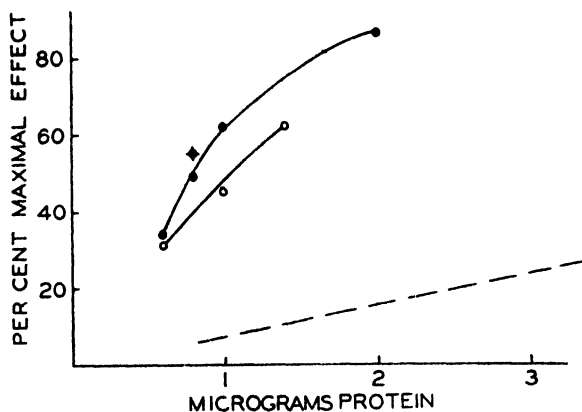
FIG. 2. Assay curves for two purified fractions of H-G factor. O, fibril filtrate of insulin after isoelectric precipitation. ●, fraction obtained from alkali-incubated insulin by ethanol and isoelectric precipitation, ◖ representing assay on another animal. The dotted line represents the activity of the amorphous insulin used as starting material.

was half maximally active at 0.8 γ. As shown below, this is not much inferior to the purification achieved by other methods, but the yield was so small that further attempts to use the fibril filtrates for purification were abandoned.

Alkali-Incubated Insulin—Fractionation of alkali-incubated insulin was attempted, since it seemed likely that the H-G factor would be more easily separated from denatured than from native insulin. It had been found previously that the H-G factor is much more resistant to alkali than insulin (6, 2). The experiment in Fig. 1 shows that about one-third of the H-G activity was lost when the insulin preparation was incubated for 2.5 hours in 0.08 N KOH at 37°. This treatment is sufficient to destroy the hypoglycemic action of insulin completely (2).

Ethanol Fractionation—A solution of alkali-incubated insulin was brought to pH 3.7 with HCl. Successive fractions were separated by addition of

ethanol and assayed for glycogenolytic activity. The results in Table I show that when the ethanol concentration reached 71 per cent a large and
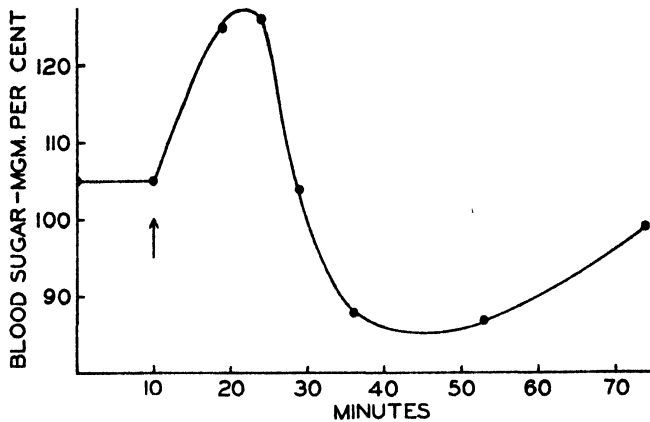
Fig. 3. Blood sugar changes following intravenous injection of partially purified insulin fibril filtrate into a rabbit. 0.22 mg. of protein was injected. An assay curve for this material on liver slices is shown in Fig. 2.

TABLE I

Fractionation of Alkali-Incubated Insulin with Alcohol

The starting material in the two experiments was (a) 100 mg. of protein in 6.5 ml. and (b) 500 mg. in 32.5 ml., both at pH 3.7.

Successive fractions	Protein recovered		Amount of protein giving half maximal activity		Activity recovered	
	(a)	(b)	(a)	(b)	(a)	(b)
	per cent	*per cent*	γ	γ	*per cent*	*per cent*
1. Ppt. from 71% ethanol	41		30		10	
2. Immediate ppt. from 83% ethanol	8	40*	10	15*	6	22*
3. Ppt. in supernatant (Fraction 2) after 1 day in cold	5	10	2	3	18	27
4. Ppt. from 85% ethanol (+ trace of KOH to incipient turbidity) after 1 day in cold	9	10	3	4	30	19
5. Ppt. from ethanol-ether	27	28	5	7	45	34
Total..	90	88			109	102

* Fractions 1 and 2 pooled for analysis and assay.

relatively inactive fraction could be separated. At 83 per cent ethanol concentration the precipitate appearing immediately also had relatively low activity, while that appearing after 1 day in the cold possessed high

activity, as did the next fraction obtained by the addition of a small amount of alcohol and alkali. The last fraction obtained by the addition of large amounts of alcohol and ether was again less active.

It may be noted in Table I that nearly one-half of the total activity was recovered in the two most active fractions (Nos. 3 and 4). These were combined and refractionated with alcohol. Table II shows that the middle

TABLE II

Refractionation of Combined Fractions 3b and 4b of Table I

The starting material for Fractions 1 to 5 was 97 mg. of protein in a volume of 11 ml., pH 3.7. Fractions 6 to 8 were obtained by further fractionation of Fraction 3.

Successive fractions	Protein recovered	Amount of protein giving half maximal activity	Activity recovered
	per cent	γ	*per cent*
1. Immediate ppt. from 83% ethanol	49	14	14
2. Ppt. in supernatant (Fraction 1) after 1 day in cold	15	6	7
3. Ppt. from 85% ethanol (+ trace of KOH to incipient turbidity)	9	1	36
4. Ppt. from 88% ethanol (+ trace of KOH to incipient turbidity)	15	2	28
5. Ppt. from ethanol-ether	11	5	9
Total	99		94
6. Immediate ppt. from 86% ethanol (+ trace of KOH to incipient turbidity)	50	1.4	36
7. Ppt. in supernatant (Fraction 6) after 1 day in cold	31	0.8	39
8. Ppt. from ethanol-ether	10	0.7	14
Total	91		89

fractions (Nos. 3 and 4) were again the most active ones and that they contained over 60 per cent of the activity. Fraction 3 on further fractionation yielded material with half maximal activity at 0.7 γ. On slow precipitation the material in these purified fractions separated in the form of tiny spheroids and flat plates.

It was noted that at this stage of purification the material lost considerable activity when standing in alcohol in the cold, and further fractionation with alcohol was therefore not attempted.

Ethanol Fractionation Followed by Isoelectric Precipitation—Fractions with half maximal activity at 2 to 3 γ of protein were obtained by precipi-

tation with ethanol. These were dissolved in 0.05 M dipotassium phosphate or 2 per cent sodium acetate, and a small amount of zinc ions was added. On addition of dilute acid to the phosphate solutions a precipitate appeared at pH 6.4. The precipitate was collected after standing 1 day in the cold. An assay showed that the amount of protein required to give half maximal activity was 0.9 γ, which represented a considerable increase in activity over the starting material. Similar results were obtained on addition of dilute acid to the acetate solutions (previously adjusted with alkali to pH 10) except that precipitation occurred at a more alkaline pH than in
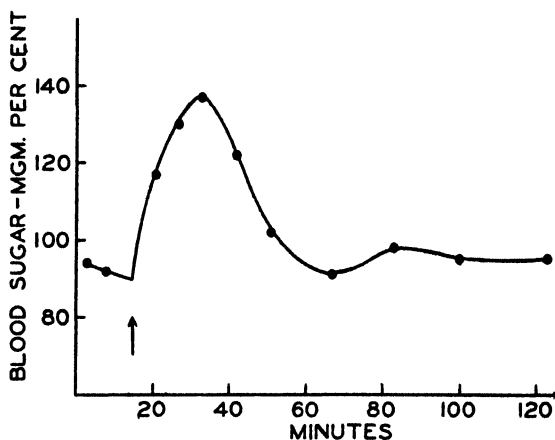
FIG. 4. Blood sugar changes following intravenous injection of H-G factor, prepared from alkali-incubated insulin, into a rabbit.   0.45 mg. of protein was injected. An assay curve for this material on liver slices is shown in Fig. 2.

the case of the phosphate solutions.   The first precipitate, appearing at pH 9.0, had less activity than the precipitates collected at pH 8.0 and pH 6.0.   All precipitates, however, were more active per unit of protein than the supernatant fluids.   An assay curve of one of the more active fractions is shown in Fig. 2 and the blood sugar response on injection into a rabbit in Fig. 4.   The blood sugar curve shows that this preparation was free of insulin.

Fractionation of Gastric Mucosa

Extraction and Precipitation by Ammonium Sulfate—Dog stomachs were used; if they could not be extracted immediately, they were kept frozen. The pyloric part was discarded and as much as possible of the muscular layers was removed from the remainder of the stomach.   The tissue was passed through a meat grinder and placed immediately in 5 volumes of 75

per cent ethanol containing 0.18 N HCl. Extraction was carried out overnight in the cold with constant stirring. Solid particles were separated by passage through gauze, and the solution was adjusted to pH 8 with ammonium hydroxide and filtered.

The alcohol was removed by evaporation with a blower and ammonium sulfate (50 gm. per 100 ml.) was added. The mixture was shaken with several portions of ether and the ether discarded. This served to remove lipide material and greatly improved subsequent filtration. Almost all the activity was found in the precipitate.

Precipitation with Alcohol-Ether and Preliminary Fractionation with Alcohol—The dissolved ammonium sulfate precipitate was dialyzed for 1 to 2 days against cold running tap water. After adjusting the pH to about 8, a variable amount of insoluble material could be discarded, but in order to

TABLE III

Ammonium Sulfate Fractionation of Gastric Factor

The starting material was half maximally active in the liver test system at about 160 γ of protein. To a solution of 470 mg. of protein in 20 ml. of water a solution of ammonium sulfate (saturated at room temperature) was added to obtain the desired degree of saturation.

Saturation of ammonium sulfate	Protein recovered	Amount of protein giving half maximal activity	Activity recov
	per cent	γ	*per cent*
0 -0.29	42	110	61
0.29–0.46	26	185	22
0.46–0.50	4	770	1

avoid losses, it was necessary to extract the residue several times with water. The factor was then precipitated by bringing the solution to pH 6 and adding ethanol to a concentration of 85 to 90 per cent, followed by the addition of an equal volume of ether. The precipitate was dried *in vacuo*, redissolved in water, and the solution brought to incipient turbidity by addition of alcohol to a concentration of 50 to 60 per cent at a pH of about 5.0. After standing 1 day in the cold, a precipitate was formed which included a brown pigment and about 25 per cent of the activity. Part of the latter could be recovered by reworking the precipitate.

The protein in solution was precipitated by addition of an excess of alcohol and ether and the precipitates were dried and pooled for further fractionation. At this stage of purification half maximal activity in the liver test system was given by 100 to 200 γ of protein. On fractionation with ammonium sulfate most of the activity was precipitated at 0.29 saturation (see Table III).

Ethanol Fractionation—About 175 mg. of the pooled and dried precipitate described in the preceding section were dissolved in 26 ml. of water at pH 3.7. As in the case of the purified pancreatic factor, the alcohol concentration had to be raised above 80 per cent before a turbidity developed. The precipitate which formed from 83 per cent alcohol overnight at 5° contained 25 per cent of the protein and 35 per cent of the activity. From the supernatant fluid a second fraction was obtained by increasing the alcohol concentration to 91 per cent and adding ether to a final concentration of 7 per cent; this fraction contained 32 per cent of the protein and 44 per cent of the activity. The remainder of the protein (43 per cent) and of the
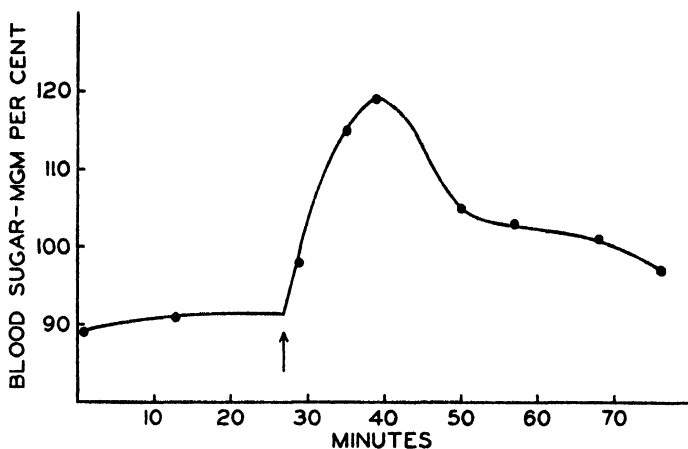
Fig. 5. Blood sugar changes following intravenous injection of H-G factor prepared from gastric mucosa into a rabbit. 1.0 mg. of protein was injected. Assay showed that this material was half maximally active in the liver test system at 27 γ of protein.

activity (21 per cent) was precipitated by adding an equal volume of ether. The first two fractions which were half maximally active in the liver test system at 130 γ were used for the fractionation described in the next section.

Isoelectric Precipitation—About 75 mg. of protein were dissolved in 17 ml. of 0.05 M dipotassium phosphate. Dilute HCl was slowly added to pH 6.2, when a slight haze developed. A small amount of zinc ions in the form of zinc acetate was added and the material kept overnight in the cold. The precipitate which formed showed half maximal activity at 27 γ of protein, while the supernatant fluid which contained the bulk of the protein was half maximally active at 130 γ. The blood sugar curve shown in Fig. 5 was obtained by injecting 1 mg. of the isoelectric precipitate into a rabbit.

In another experiment an isoelectric precipitate was obtained at pH 6.2 which was half maximally active at 12 γ of protein. This was the most active fraction prepared from gastric mucosa, but further purification was precluded by the small amount of material left.

Trichloroacetic Acid Precipitation—A further similarity between the pancreatic and gastric factors is that both can be fractionated with trichloroacetic acid (TCA). In the case of alkali-incubated insulin a considerable part of the factor was precipitated by 1.5 per cent TCA, and the remainder was precipitated when the concentration of TCA was raised to 4.5 per cent. Similar results with the gastric factor are shown in Table IV.

Electrophoretic Patterns—The amorphous insulin (*A*) and the crystalline zinc insulin (*B*), which were used for the purification of the H-G factor,

TABLE IV

Fractionation of Gastric Factor with Trichloroacetic Acid

The starting material was half maximally active in the liver test system at about 100 γ of protein. 18 per cent trichloroacetic acid (TCA) was added to a solution of 45 mg. of protein in 12.5 ml. of water.

Successive fractions	Protein recovered	Amount of protein giving half maximal activity	Activity recovered
	per cent	γ	*per cent*
1. 1.5% TCA ppt.	12	58	21
2. 3.0% " "	18	52	35
3. 6.0% " "	18	130	14
4. Ppt. of supernatant (Fraction 3) from ethanol-ether	19	195	10
Total....	67		80

were investigated in the Tiselius apparatus equipped with a long center section and the cylindrical lens optical system. A sample of Novo[*] insulin (*C*) which was free of the H-G factor is included for comparison. Acetate buffer of ionic strength 0.1 and at pH 3.8 was used (Fig. 6).

The mobilities for the main peak on the ascending side were (in sq. cm. volt^{-1} sec.$^{-1}$ × 10^5 at 1.9°) 5.27, 5.18, and 5.27 for *A*, *B*, and *C*, respectively. Measurements on the descending side gave values which were in fair agreement. The descending boundaries were much more spread out than the ascending ones.

On the ascending side (Fig. 6) one sees a small, very slow moving peak in all three insulin preparations. In one experiment with crystalline zinc insulin this peak was separated from the main component in the electro-

---

[*] Obtained from the Novo Terapeutisk Laboratorium, Denmark.

phoresis cell by pushing it by means of the compensator device above the plate separating the U tube on the descending side. The amount of protein recovered was 0.4 mg. (out of 40 mg. present at the start of the run) and there was no concentration of the H-G factor in this fraction. A separation of part of the main component on the ascending side yielded 32 mg. of protein, while the U tube yielded 8 mg. The H-G factor was found to be associated with the main component. A repetition of this experiment with amorphous insulin, in which case a smaller amount of protein was cut off on the ascending side, again showed that the H-G factor was migrating with the main component.
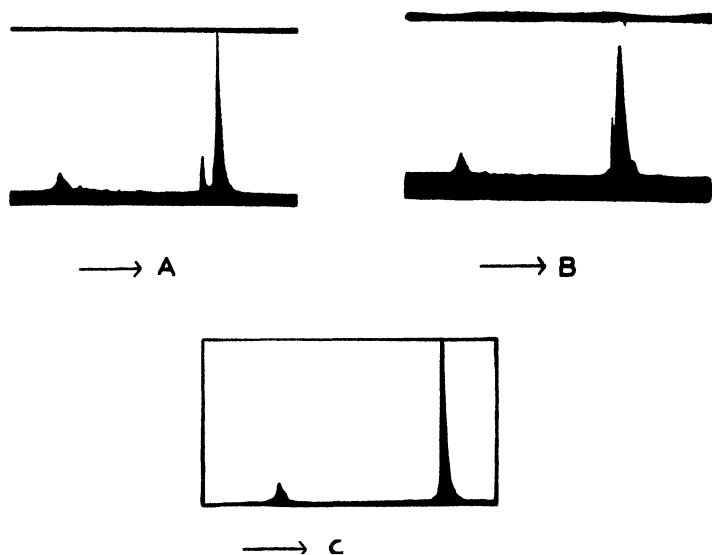
FIG. 6. Electrophoretic schlieren diagrams of ascending boundaries in acetate buffer, pH 3.8, ionic strength 0.1, after 4 hours at 1.9°. (A) amorphous insulin, (B) crystalline zinc insulin, (C) Novo insulin free of hyperglycemic factor.

It may be seen in Fig. 6 that the electrophoretic patterns of the three insulin preparations differ in that the amorphous and the crystalline zinc insulin, but not the Novo preparation, show a small peak just behind the main protein component. This peak can be detected after 1 hour of electrophoresis at pH 3.8 and is fairly well separated, particularly in the amorphous preparation, after 4 hours of electrophoresis. This peak may be tentatively identified as the H-G factor, since it is absent in the Novo preparation. An estimate of the area under this peak on an enlarged projection gave 14 per cent for (A) and 10 per cent for (B). This may be compared with the 10-fold increase in H-G activity per mg. of protein on isolation of the H-G factor from the same insulin preparations.

DISCUSSION

The destruction of activity of the H-G factor from pancreas or from gastric mucosa by proteolytic enzymes (1) and the fact that the materials can be dialyzed without loss and purified by protein fractionation methods point to the protein nature of the H-G factor. The H-G factor present in the upper two-thirds of the gastric mucosa of the dog can be purified by the same methods of extraction and precipitation that were successful in the purification of the H-G factor of pancreatic origin, an indication that the two factors are closely related if not identical. A search of the literature (see the previous review (1)) has provided a histological basis for these findings. Similar cell types which stain characteristically with silver showed a distribution in the gastrointestinal tract and in the pancreas which followed the distribution of the H-G factor. In the pancreas these argentophil cells have been identified by some authors as the α cells and experimental evidence has been presented that the H-G factor may originate in the α cells (1).

Some commercial insulin preparations, including crystalline zinc insulin, seem to contain 5 to 10 per cent of the H-G factor. This preliminary estimate is based partly on electrophoretic observations, partly on an about 10-fold increase in H-G activity per mg. of protein on purification of the H-G factor from insulin. The tiny spheroids and flat plates described in a preceding section were half maximally active in the liver test system at 0.7 γ of protein, while between 6 and 7 γ of insulin were required to give the same effect. On intravenous injection of H-G factor free of insulin into rabbits, marked effects on the blood sugar level are obtained with 0.2 mg. of protein (the minimal amount necessary for a distinct blood sugar rise was not determined). No further information is available at this time on a possible physiological rôle of this factor; its mechanism of action in producing glycogenolysis in the liver is under investigation.

SUMMARY

1. Methods are described for the purification of the hyperglycemic-glycogenolytic factor of the pancreas and of the gastric mucosa of the dog. Both factors showed essentially the same behavior during various procedures used in their isolation, such as dialysis, salting out with ammonium sulfate, precipitation with trichloroacetic acid, alcohol fractionation, and isoelectric precipitation. The two factors are protein in nature. They appear to be closely related and they have been prepared free of insulin. On intravenous injection into rabbits they give the same blood sugar response.

2. A highly purified amorphous insulin, or crystalline zinc insulin, was used as the source of the pancreatic factor. When insulin fibrils were formed

by the method of Waugh and then removed by filtration, the filtrate was found to contain an increased amount of the H-G factor per mg. of protein. Further purification resulted in about a 9-fold increase in activity per mg. of protein, as determined with the liver test system. Fractionation of alkali-incubated insulin resulted in a 10-fold increase in specific activity and yielded a preparation in the form of tiny spheroids which was free of insulin.

3. A separate protein component has been detected in electrophoretic patterns of amorphous insulin and of crystalline zinc insulin in acetate buffers at pH 3.8. This component amounted to about 10 per cent of the total protein and migrated at a slightly slower rate than insulin. Preparations of insulin which were free of the hyperglycemic-glycogenolytic factor did not show this component. From these observations and from the degree of purification achieved, it may be concluded that various insulin preparations contain about 5 to 10 per cent of another protein in the form of the hyperglycemic-glycogenolytic factor.

The technical assistance of Miss Dolores Barta is gratefully acknowledged. The authors also wish to thank Mr. Robert Loeffel for carrying out the electrophoretic measurements.

BIBLIOGRAPHY

1. Sutherland, E. W., and de Duve, C., J. Biol. Chem., 175, 663 (1948).
2. Sutherland, E. W., and Cori, C. F., J. Biol. Chem., 172, 737 (1948).
3. Kabat, E. A., and Mayer, M. M., Experimental immunochemistry, Springfield (1948).
4. Weichselbaum, T. E., Am. J. Clin. Path., 16, Tech. Suppl., 10, 40 (1946).
5. Waugh, D. F., J. Am. Chem. Soc., 68, 247 (1946).
6. Burger, M., and Brandt, W., Z. ges. exp. Med., 96, 375 (1935).

THE UTILIZATION OF SOME PHENYLPYRUVIC ACIDS FOR GROWTH IN THE RAT*

By EDWARD C. BUBL AND JOSEPH S. BUTTS

(*From the Department of Agricultural Chemistry, Oregon Agricultural Experiment Station, and the Biochemical Laboratory of the Department of Chemistry, Oregon State College, Corvallis, Oregon*)

Throughout the normal pathway of the metabolism of most amino acids there is a constant loss or interchange of amino groups. In the process of deamination keto acids, rather than hydroxy acids, appear to be the chief products (1, 2). In this manner a variety of keto acids are continuously being generated in the animal body.

The fate of these naturally occurring carbonyl compounds is of particular interest in their relation to the promotion of growth. The keto analogue has been reported as capable of substituting for the amino acid in the case of methionine (3), tryptophan (4), histidine (5), leucine, isoleucine (6), and phenylalanine (7). Reports concerning the remaining essential amino acids have not appeared in the literature.

Tyrosine, usually considered as a non-essential amino acid for the growth of the white rat, has been shown by Womack and Rose (8) to be capable of replacing about one-half of the phenylalanine requirement of the growing rat. Since tyrosine is non-essential in the presence of sufficient phenylalanine, the growth effect of the analogues has never been established.

In an earlier paper (9) it has been shown that when suboptimal amounts of phenylalanine are incorporated in an amino acid mixture, otherwise adequate for the white rat, the additional requirement for phenylalanine is met equally well with D-, L-, or DL-tyrosine, as evidenced by normal growth.

By means of this technique we have studied the utilization of the keto analogue of tyrosine, *p*-hydroxyphenylpyruvic acid, for growth in the rat.

The keto analogue of phenylalanine, phenylpyruvic acid, has been stated in a review by Rose (7) to be capable of promoting growth in the absence of phenylalanine. As far as we are aware, no experimental data were reported.

Neither confirmation nor denial of this statement has appeared subsequently in the literature. Therefore, this analogue also was tested for its ability to replace phenylalanine in the diet of the growing rat.

EXPERIMENTAL

Weanling rats of the Evans-Long strain were used in this study. They were distributed as evenly as possible with respect to weight, litter, and sex. At 28 days of age the animals were placed in individual cages and were

TABLE I

Composition of Amino Acid Mixture

	gm.
Glycine... .	0.1
Alanine .	0.7*†
Serine .	0.2*
Valine . .	2.0*
Leucine . .	2.4*
Isoleucine	1.6*
Cystine	0.2
Methionine	0.8*
Threonine . ..	1.4*
Tryptophan	0.4*
Aspartic acid . ..	0.2
Glutamic "	2.0
Lysine monohydrochloride	3.0*
Proline . ..'	0.2
Histidine monohydrochloride	0.95
Arginine	0.60
Sodium bicarbonate .	1.76
	18.51

* Racemic acid.

† Alanine was increased when keto acids were fed to keep the nitrogen content constant

allowed water and the experimental diet *ad libitum*. Weights were recorded daily.

The basal diet and the vitamin supplements were made up according to Borman *et al.* (10), with the exception that biotin was omitted. It was assumed and later demonstrated (9) that, since liver extract was included, this omission would not be a limiting factor in a 28 day experiment. The animals were fed daily from a weighed amount of food in the containers. The food thus measured was always in excess of that consumed. The food intakes were calculated by difference after careful collection of any spillage.

The amino acid mixture was prepared from crystalline amino acids from

several sources. In every case these compounds gave excellent analytical values, indicating a high degree of purity.

The amino acid mixture shown in Table I was patterned after that of Borman *et al.* (10), except that phenylalanine and tyrosine were omitted and arginine was added. Hydroxyproline was also omitted, since earlier studies have shown (11, 9) that this omission was not critical. Alanine was added

TABLE II

Experiments Showing Weight Gain with Various Supplements Added to Basal Diet

Each experiment covered 28 days.

Litter No.	Rat No. and sex	Total gain in weight	Total food intake	Supplements
		gm.	*gm.*	
31	106 ♂	34	135	1.0% phenylpyruvic acid,
31	107 ♀	26	131	0.5% DL-alanine
31	108 ♂	40	155	
33	114 ♂	33	149	
33	115 ♀	37 (34)*	149	
31	103 ♂	35	131	0.5% DL-phenylalanine,
31	104 ♂	38	164	0.5% *p*-hydroxyphenyl-
31	105 ♀	34	152	pyruvic acid, 0.25% ala-
33	118 ♂	34	175	nine
33	119 ♂	34	179	
33	120 ♀	39 (35.6)	172	
32	109 ♀	−8	142	0.5% DL-phenylalanine,
32	110 ♀	7 (−0.5)	124	0.25% DL-alanine
32	111 ♂	Died (25 days)	82	
30	101 ♂	−10	99	None
30	102 ♂	−11	93	
33	117 ♂	−6	68	
33	121 ♀	−4 (−7.7)	64	
6	37 ♀	46	177	0.5% DL-phenylalanine,
6	38 ♂	47	199	0.5% L-tyrosine (Bubl and
6	39 ♂	43	188	Butts (9))
7	85 ♀	32 (42)	196	

* The figures in parentheses denote the average for group.

to all the experimental diets to compensate for any lowered nitrogen content. The basal diet containing the amino acid mixture, but without tyrosine and phenylalanine or the keto analogues, was made up at frequent intervals to prevent the development of rancidity.

Phenylpyruvic acid was prepared according to Herbst and Shemin (12). After recrystallization from chloroform the compound yielded the following analytical data.

Calculated, C 65.85, H 4.8; found, C 65.91, H 4.87

The p-hydroxyphenylpyruvic acid was prepared in an analogous manner with p-hydroxybenzaldehyde as starting material. Analysis of the product after recrystallization from chloroform was as follows:

Calculated, C 55.20, H 4.08; found, C 55.05, H 4.17

The compounds were prepared every few days inasmuch as they are relatively unstable.
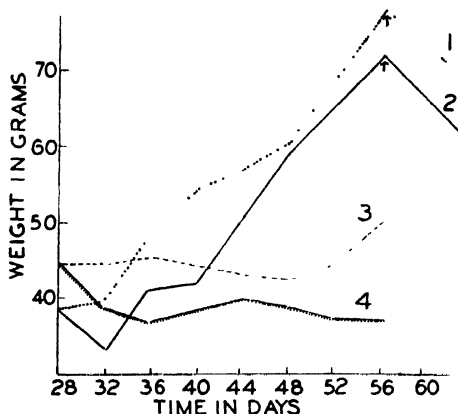
Fig. 1. Growth response of 28 day-old rats to a basal diet containing the keto analogues of tyrosine and phenylalanine. Curve 1, basal diet + 0.5 per cent phenylalanine + 0.25 per cent alanine + 0.5 per cent p-hydroxyphenylpyruvic acid; Curve 2, basal + 1.0 per cent phenylpyruvic acid + 0.5 per cent alanine; Curve 3, basal + 0.5 per cent phenylalanine + 0.25 per cent alanine; Curve 4, basal + 0.5 per cent alanine. The arrows indicate the point at which the keto acids were withdrawn from the diet.

DISCUSSION

Using a technique similar to that of Womack and Rose (8), we have been able to determine the growth-promoting capacity of the keto analogues of both tyrosine and phenylalanine. It can be seen from Table II that if all the phenylalanine is removed from the diet, and no supplement is added, loss in weight results. If the phenylalanine-free diet is supplemented with 1.0 per cent phenylpyruvic acid, the animals gain in weight and their appearance is excellent.

These experiments confirm the statement of Rose (7) that the keto analogue can totally replace its parent amino acid, phenylalanine. It also corroborates the work of Moss (13) who showed that ingested deuterophenyllactic acid forms tyrosine in the normal rat. Moss concluded that the most likely pathway for this conversion is through phenylpyruvic acid, followed by oxidation on the ring, then amination to tyrosine.

When the basal diet was supplemented with 0.5 per cent phenylalanine

and 0.25 per cent alanine, poor growth resulted (Table II). When the above diet was supplemented with 0.5 per cent p-hydroxyphenylpyruvic acid, excellent growth was obtained. Apparently sufficient nitrogen is present to allow animation of the compound.

Fig. 1 shows the effect of withdrawing the keto acid supplements from the diet. Two rats from each group were continued for an additional 8 day period. With all the animals the response was the same, a loss of 9 to 11 gm. during this period.

During the first 10 to 12 days, the animals receiving the diets supplemented with the keto acids consumed much less food than those receiving phenylalanine or tyrosine supplements; consequently growth was much inferior. After this initial period, the animals increased their food consumption and good growth resulted. However, the loss was not fully regained and consequently the total growth appears inferior. If one were to take as an average the gain from the 40th to the 56th day, the total would be well above the average gains of the animals receiving 1.00 per cent phenylalanine (9). The poor food intake is undoubtedly the reason for the slow gains, but the reason for the poor food intake is unknown.

SUMMARY

1. The keto analogue of tyrosine, p-hydroxyphenylpyruvic acid, can replace tyrosine required to meet the demands of the rat on an amino acid mixture suboptimal in phenylalanine.

2. Phenylalanine can be replaced completely by its keto analogue phenylpyruvic acid in the amino acid requirement of the growing rat.

3. Because of poor food intakes the animals lose weight or fail to grow during the first 8 to 10 days of the experiment. However, as soon as the food intakes reach normal levels, the animals grow at excellent rates.

BIBLIOGRAPHY

1. Bernheim, F., Bernheim, M. L. C., and Gillaspie, A. G., J. Biol. Chem., 114, 657 (1936).
2. Borek, E., and Waelsch, H., J. Biol. Chem., 141, 99 (1941).
3. Cahill, W. M., and Rudolph, G. G., J. Biol. Chem., 145, 201 (1942).
4. Jackson, R. W., J. Biol. Chem., 84, 1 (1929).
5. Harrow, B., and Sherwin, C. P., J. Biol. Chem., 70, 683 (1926).
6. Rose, W. C., Physiol. Rev., 18, 109 (1938).
7. Rose, W. C., Science, 86, 298 (1937).
8. Womack, M., and Rose, W. C., J. Biol. Chem., 166, 429 (1946).
9. Bubl, E. C., and Butts, J. S., J. Biol. Chem., 174, 637 (1948).
10. Borman, A., Wood, T. R., Wood, H. C., Anderson, E. G., Oesterling, M. J. Womack, M., and Rose, W. C., J. Biol. Chem., 166, 585 (1946).
11. Womack, M., and Rose, W. C., J. Biol. Chem., 171, 37 (1947).
12. Herbst, R. M., and Shemin, D., Org. Syntheses, coll. 2, 519 (1943).
13. Moss, A. R., J. Biol. Chem., 137, 739 (1941).

THE BIOLOGICAL FORMATION OF SERINE FROM GLYCINE*

By PHILIP SIEKEVITZ AND DAVID M. GREENBERG

(*From the Division of Biochemistry, University of California Medical School, Berkeley*)

The biochemical relationship of glycine to serine was first shown by Shemin, who, in 1946, demonstrated the conversion *in vivo* of serine to hippuric acid, thus to glycine (1). The reverse of this reaction, the synthesis of serine from glycine, has been observed by Goldsworthy, Winnick, and Greenberg *in vivo* (2), by Winnick, Moring-Claesson, and Greenberg in rat liver homogenates (3), and by Siekevitz, Winnick, and Greenberg in rat liver slices (4). Recently, Sakami (5) has demonstrated *in vivo* that added formate condenses with the α-carbon of glycine to form serine. This is the reverse of the mechanism postulated by Shemin for the serine to glycine reaction. If this is a normal metabolic reaction, the question immediately arises as to the origin of this formate. It may be noted that Sonne, Buchanan, and Delluva (6) have shown that formate can be utilized in the pigeon in the synthesis of uric acid.

A clue to the origin of formate and to the mechanism of the glycine-serine reaction was obtained when it was discovered that the rate of uptake of the label from α-labeled glycine into the protein of rat liver slices was nearly 1½ times greater than the uptake when carboxyl-labeled glycine was used (7). This indicated a difference in the metabolic pathways between the two carbon atoms of glycine. When the serine was isolated both from the protein hydrolysates and from the trichloroacetic acid filtrates of these slices, it was noted that the distribution of the radioactivity was dependent upon the position of the label in the added glycine (7). With carboxyl-labeled glycine, only the carboxyl carbon of serine was found to be radioactive; with α-labeled glycine, both the α- and β-carbons of serine were active. Therefore, since the α-carbon of glycine may form the β-carbon of serine and since Sakami has shown that formate goes to the β-carbon of serine in the presence of glycine, it was desirable to determine whether the α-carbon of glycine is converted to formate. The probability of finding the latter reaction to be a correct one is en-

hanced by the recent observation of Karlsson and Barker (8) that the α-carbon of glycine, as well as formate, is involved in the formation of carbon atoms 2 and 8 of uric acid in the pigeon.

General Methods—The rats used were of the Wistar-Slonaker strain, of about 200 gm. weight, previously fasted for 24 hours. The livers were excised and slices were cut and placed in Krebs-Ringer phosphate buffer of pH 7.4, which had been previously gassed with oxygen or with nitrogen for 10 minutes. In all experiments, fifteen slices, totaling approximately 750 mg., wet weight, were placed in 10 ml. of medium. They were incubated with the specified radioactive substrate for 3 hours at 37°. The anaerobic experiments were carried out in an atmosphere of nitrogen, while the aerobic experiments were conducted in air.

All the radioactive samples were deposited on filter paper (Whatman No. 50), prepared as described by Melchior and Tarver (9), and were counted by using a thin mica window tube and a Tracerlab autoscaler model Geiger-Müller counter. The over-all efficiency of the counting arrangement was 6 per cent. The areas of the samples were uniformly 12.25 sq. cm. The counting time was adjusted to give a counting error of from 2 to 3 per cent. The radioactive material could be scraped off the hard filter paper easily and reproducibly, so that the weight of the material was obtained by weighing the filter paper before and after scraping off the sample.

Isolation of Serine—In the experiments in which only serine was isolated, 10 per cent trichloroacetic acid (TCA) was added at the end of the incubation period to make a final concentration of 5 per cent. The tissue was homogenized, centrifuged, and the supernatant drawn off. The latter was extracted with ether to remove the TCA, and the solution concentrated to a small volume. Carrier serine was then added, varying from 400 to 800 mg., the amount being dependent upon the number of purification steps which had to be carried out. The solution was then neutralized, and 5 volumes of alcohol were added to precipitate the serine. This was then recrystallized five times from water, the p-toluenesulfonyl derivative prepared (10), and the solution recrystallized twice from 60 per cent alcohol. The serine was counted in the form of this derivative, and the specific activity corrected to the weight of the amino acid. The melting points of the derivative (212–213°) agreed with that given in the literature. The C^{14} of the carboxyl carbon was determined by treatment of the serine with ninhydrin (11), the CO_2 liberated being caught in $Ba(OH)_2$ and counted as $BaCO_3$. The β-carbon of serine was obtained by the periodate method of Nicolet and Shinn, as modified by Rees (12). The formaldehyde re-

leased by this treatment was steam-distilled into dimedon, and the condensation product thus obtained (methylenebis methone) was recrystallized twice from water by alternately acidifying and then making the solution alkaline. This derivative was counted as such. The melting point of the methylenebis methone checked well with the value given in the literature (189°).

Isolation of Glycine—In experiments in which glycine was isolated, a definite amount of carrier glycine was added to the ether-extracted TCA filtrates, the solution was neutralized, and 5 volumes of alcohol were added to precipitate the glycine. This was recrystallized twice from aqueous alcohol and the acetyl derivative of the glycine prepared (13) and recrystallized twice from water for counting (m.p. 207–208°). The acetyl glycine was then hydrolyzed with 1 N HCl to give free glycine. The latter was then recrystallized to constant specific activity. Both the carboxyl and α-carbon atoms were obtained from the glycine by the ninhydrin method. After the CO_2 from the carboxyl carbon had been caught in $Ba(OH)_2$ and counted as $BaCO_3$, the residual solution was distilled to dryness, a water condenser being employed, with a 0.4 per cent dimedon solution placed in the receiver, in order to catch the formaldehyde from the α-carbon. The distillation to dryness was repeated three times, water being added each time. This treatment gave an adequate precipitate of the methylenebis methone, which was then recrystallized and counted.

Isolation of Formate—In those experiments in which formate was to be isolated, formate at twice the molar concentration of the glycine was added at the beginning of the experiment in order to trap any of this compound containing C^{14} that might be produced. The pH of the incubation mixture was then adjusted to 7.4, and, at the end of the 3 hour period, 60 mg. of formic acid were added as carrier. The protein was then denatured by heating, and the tissue homogenized and centrifuged. The supernatant solution was then steam-distilled until 15 volumes came over. This quantity was enough to obtain about half of the formate originally added as carrier. The steam distillate was next made alkaline, evaporated to dryness, taken up in carbon dioxide-free water, acidified, and any residual carbon dioxide driven off by 20 minutes of boiling and aerating. The formate was then oxidized to carbon dioxide by mercuric oxide (14) and the carbon dioxide caught as barium carbonate and counted as such. This method is considered to be specific for formate, and in our hands acetate, pyruvate, and glyoxylate were not oxidized to carbon dioxide by the procedure.

The solution which remained after the steam distillation was evaporated to a small volume, filtered, and subjected to the carrier method of analysis, as already described for serine and glycine.

Control Experiments—For the methods given above to be considered valid, it must be shown that (1) the specific activity of a given amino acid is not changed by its conversion into derivatives or degradation products if the specific activity is expressed in terms of the weight of the amino acid, and (2) that the compounds isolated by the carrier technique are free of radioactive impurities.

In dealing with the first criterion it has been pointed out (15) that "in practice all carbon compounds that do not contain atoms of high atomic number require practically identical correction (for self-absorption) at any given surface density, all other variables being the same." Accordingly, a table of self-absorption corrections obtained with radioactive glycine[1] was used for various carbon compounds containing elements of about the same atomic weight.[2]

The following experiment was designed to show that the specific activity of the α-carbon of glycine (counted as the methylenebis methone derivative) plus that of the carboxyl carbon (counted as $BaCO_3$) is equal to that of glycine (counted as glycine) and to that of acetyl glycine (counted as acetyl glycine), when each specific activity is expressed in terms of glycine.

Carboxyl-labeled glycine was mixed with α-labeled glycine to give, in effect, doubly labeled glycine. To an aliquot of this preparation carrier glycine was added, and the product was precipitated by alcohol and counted as glycine. The ninhydrin method already described was used to isolate both carbon atoms of the glycine. The carboxyl carbon was counted as $BaCO_3$, the α-carbon, which gives formaldehyde by this method, as the HCHO-dimedon compound, after recrystallizing twice from water. Another portion of the carrier glycine was converted to the acetyl derivative (see above) and counted. This acetylglycine was treated as before to give free glycine, and the C^{14} of the carboxyl and α-carbons of the glycine obtained from acetylglycine was determined as described above. The results are given in Table I. The specific activity of the acetylglycine was multiplied by the factor 1.56, which is the ratio of the molecular weight of acetylglycine to that of glycine. Likewise, the activity of the HCHO-dimedon compound was multiplied by 3.89 (the ratio of the molecular weight of this derivative to that of glycine). The specific activity of the $BaCO_3$ was multiplied by 2.63 (the ratio of the molecular weight of $BaCO_3$ to glycine). The self-absorption corrections for $BaCO_3$ were taken from Table I given by Yankwich and coworkers (16). The absorption curve

---

[1] Goldsworthy, P., Greenberg, D. M., Peterson, E., Siekevitz, P., and Winnick, T., unpublished experiments.

[2] In the case of the *p*-toluenesulfonyl derivative of serine, referred to later, the sulfur contained in the molecule is considered insufficient to alter significantly the self-absorption correction obtained from the glycine curve.

for glycine was used for glycine, acetylglycine, and the HCHO-dimedon derivative.[1]

It can be seen that, within a 3 per cent error, both the glycine figures agree with that for acetylglycine. The sum of the values for the α- and carboxyl carbons of glycine agrees within 4 per cent with the corresponding glycine figures.

The second criterion, that of the absence of radioactive contaminants, is a more serious one, since a very minute quantity of radioactive impurity, if it is fairly active, can produce a large error in the specific activity of the isolated compound. It has been shown (3) that serine can be freed from traces of radioactive glycine by repeated recrystallizations. Be-

TABLE I

Specific Radioactivity of Glycine, As Determined by Counting Glycine and Its Derivatives

Compound isolated	Specific activity	Specific activity as glycine	Average specific activity as glycine
	counts per min. per mg.	counts per min. per mg.	counts per min. per mg.
Original glycine..	83.2	83.2	
	83.0	83.0	83.1
BaCO₃ from carboxyl C	11.9	31.3	
	14.7	38.7	34.0
Methylenebis methone from α-C	11.9	46.3	46.3
Acetylglycine	51.5	81.5	81.5
Glycine from hydrolysis of acetylglycine	83.2	83.2	
	84.1	84.1	83.6
BaCO₃ from carboxyl C	12.7	33.4	33.4
Methylenebis methone from α-C ..	11.4	44.3	
	12.3	47.8	46.1

cause this procedure is critical in the carrier technique, those experiments were repeated, with the added step of converting the serine to the p-toluenesulfonyl derivative. Table II shows that this procedure is sufficient to rid the serine of traces of radioactive glycine impurity.

The reverse experiment, *i.e.* the separation of traces of radioactive serine from glycine, could not be performed because of the unavailability of radioactive serine. However, the specific activity of glycine obtained from radioactive protein hydrolysates, which also contained radioactive serine, was not altered upon acetylation and recrystallization (2).

For the HCHO-dimedon derivative, constant specific activity was held a sufficient criterion of purity, provided that the formaldehyde was steam-distilled into the dimedon solution. In certain experiments, when the

dimedon solution was added directly to the reaction mixture, the precipitated HCHO-dimedon derivative had activity. These could not be removed by two recrystallizations from water, although the specific activity did drop. If, however, a change of phase was made to occur (steam dis-

TABLE II

Purification of Serine Containing Radioactive Glycine

0.1 mg. of glycine (40,000 counts per minute) was added to a 1 ml. solution containing 800 mg. of serine.

Purification process	Specific activity
	counts per min. per mg.
Initial serine (calculated)....	50.0
Crystallized once from alcohol..	26.1
Recrystallized twice more from alcohol and once from water	13.9
Recrystallized once more from water	4.2
" " " " "	1.6
p-Toluenesulfonyl derivative prepared and recrystallized once from alcohol......	0.2
Derivative recrystallized once more from alcohol ..	0.0

TABLE III

Formation of Serine from Glycine

1 mg. of radioactive glycine was used in each experiment. The radioactivity of the glycine varied in the individual experiments, ranging from 2 to 3 μc.

Experiment No.*	Position of C^{14} in glycine	Atmosphere and additions	Recovered counts of serine†			
			Total serine	α-C‡	β-C	Carboxyl C
1a	α-C	Air	4.2	2.9	1.3	0.0
1b	Carboxyl C	"	2.9	0.0	0.0	2.9
2	" "	N	5.5			
3	" "	"	5.8			
4	" "	"	5.6			
5a	α-C	"	7.7	7.4	0.3	0.0
5b	"	Air; 0.01 M KCN	7.8			

* Each experiment refers to a different animal.

† Per cent recovered counts (see equation (1) for the definition).

‡ The per cent of counts in the α-carbon equals the total minus that in the β-carbon minus that in the carboxyl carbon.

tillation of the formaldehyde), activity of the HCHO-dimedon derivative could no longer be counted.

RESULTS AND DISCUSSION

Table III summarizes the experiments on the aerobic and the anaerobic synthesis of serine from glycine by rat liver slices. Table IV gives the

detailed data of a typical experiment (Experiment 1a in Table III). These data are representative of the procedures employed in the experiments in Table III and of the results reported in Tables IV to IX. To calculate the per cent of recovered counts, it must be first noted that the over-all

TABLE IV

Representative Experimental Data on Formation of Serine from Glycine

Fifteen rat liver slices were incubated for 3 hours in 10 ml. of Krebs-Ringer phosphate buffer of pH 7.4 in each of five flasks, to which was added 1 mg. (2.88 μc.) of $C^{14}H_2NH_2COOH$ to give a final concentration of 1.2 mM per liter. The medium was aerated with O_2 for 10 minutes previous to the addition of the slices. The TCA filtrates of these five flasks, containing a total of 1,926,000 counts per minute were pooled, and to three-fourths of this solution, containing 1,445,000 counts per minute, were added 1000 mg. of serine carrier.

Serine derivative and isolation procedure employment	Specific activity as serine	Recovered counts
	counts per min. per mg.	
Serine recrystallized once from water....	105.6	
" " twice more from water	100.2	
p-Toluenesulfonyl derivative, recrystallized once from alcohol	82.8	
" " " " more from alcohol . ..	64.1	
p-Toluenesulfonyl derivative, recrystallized once more from alcohol*	61.4	4.2
HCHO-dimedon compound, from β-C of serine	25.9	
" " recrystallized once from water	22.4	
" " " " more from water†	19.4	1.3
BaCO$_3$ from carboxyl C of serine...	0.0	0.0

* M.p. 212–213°; reported by MacChesney and Swann (10) 212–213°; N 5.56 per cent, calculated 5.43 per cent.
† M.p. 189–190°; reported by Rees (12) 189°.

efficiency of the counting arrangement is 6 per cent, and that 1 μc. is equal to 2.33×10^6 counts per minute. The formula used, therefore, is

(1) $\quad$ Recovered counts $= 100$

$$\times \frac{\text{specific activity of radioactive compound} \times \text{amount carrier added}}{\text{total counts added } (2.33 \times 10^6 \times 0.06 \times \text{microcuries added})}$$

It can be noted (Table III) that the glycine to serine reaction goes at a faster rate in nitrogen than in air,[3] and that cyanide has no inhibiting

[3] One result contrary to this finding is given in Table VI (Experiments 4a and 4b). No explanation can now be offered for this exception.

effect. It is seen that the α-carbon of glycine forms both the α- and β-carbons of serine, while the carboxyl carbon forms only the carboxyl carbon of serine. Also, Experiments 1a and 1b, taken together, indicate that the α- and carboxyl carbons of the glycine go together to form serine, since the percentage of recovered counts (2.9) is the same in the two cases.

To demonstrate this in another way, doubly labeled glycine was used, as described in the experimental section. The serine was isolated from the TCA filtrates and the C^{14} distribution was determined. The percent-

TABLE V

Formation of Labeled Serine from Non-Radioactive Glycine and Radioactive Formate

1 ml. of radioactive sodium formate, equivalent to 0.6 mg. of formic acid and containing 333,000 counts per minute, was added to make a final concentration of 1.2 mM per liter. The glycine concentration was 1.2 mM per liter. Glycine was also isolated and gave zero counts in the intact glycine and in the α- and carboxyl carbons in these experiments.

Experiment No.*	Atmosphere	Recovered counts of serine			
		Total	α-C	β-C	Carboxyl C
1a	Air	3.70	0.00	3.70	0.0
2a	"	3.04	0.01	3.03	0.0
1b	N	0.44	0.11	0.33	0.0
2b	"	0.11	0.01	0.10	0.0

* Each experiment number refers to a different animal.

age distribution of the radioactivity present in each of the carbon atoms is given below.

$$55\% \text{—} CH_2NH_2 \qquad 14\% \text{—} CH_2OH$$
$$45\% \text{—} COOH \quad \rightarrow \quad 44\% \text{—} CHNH_2$$
$$42\% \text{—} COOH$$
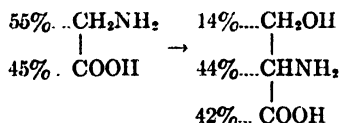
This distribution indicates that 1 molecule of glycine goes to serine intact, while another molecule is broken up and the α-carbon attaches to the α-carbon of the unsplit glycine to form the β-carbon of the resulting serine. This result raises the question: In what form is the α-carbon of glycine transferred to become the β-carbon of serine?

Synthesis of Serine from Formate and Glycine—First, the Sakami experiment was repeated with our *in vitro* procedure. Radioactive formate was added to an equimolar amount of non-radioactive glycine and the glycine and serine were isolated. The results are reported in Table V. It can be seen that Experiments 1a, 2a, and 2b in Table V agree with those of Sakami in that all the radioactivity can be accounted for in the

β-carbon. However, in Experiment 1b only 75 per cent of the total serine C^{14} can be accounted for in the β position. No counts were found in the carboxyl group in this instance. The discrepancy may well be due to a counting error because of the low specific activity of the serine (1.6 counts per minute per mg.) and that of its β-carbon (1.2 counts per minute per mg.).

The formate fixation proceeds more rapidly in air than in nitrogen. This can mean either that the fixation requires oxygen or else that there is a greater dilution of the formate anaerobically.

Degradation of Glycine to Formate—The results in Table VI show that formate is produced from the α-carbon of glycine and not from the car-

TABLE VI

Production of Formate from Glycine

1 mg. of radioactive glycine was added in each experiment to make a final concentration of 1.2 mM per liter.

Experiment	Position of C^{14} in glycine	Concentration of added formate	Atmosphere	Recovered counts		
				Serine		Formate
				Total	β-C	
		mM per l.				
1	α-C	2.4	Air	7.9	1.09	0.88
2a	"	2.4	N	10.2	0.50	0.36
2b	Carboxyl C	2.4	"			0.00
3a	α-C	2.4	"			0.25
3b†	"	2.4	Air			0.00
4a	"	1.2	"	7.9	1.31	0.69
4b	"	1.2	N	5.4	0.27	0.25

* Each experiment refers to a different animal.
† The tissue was boiled in this experiment, then added to the medium.

boxyl carbon. In Experiment 3b, in which boiled slices were used, the CO_2, which was the oxidation product of the distilled formate, had no radioactivity.

The data in Table VII, which is a composite of certain of the results in Tables III and VI, give added evidence for the formation of formate from the α-carbon of glycine. It is to be noted that in air, but not in nitrogen, the C^{14} in the β-carbon of serine is diluted in those experiments in which inactive formate was added. A possible explanation of this is that there is a greater amount of inactive formate already available in the presence of nitrogen, so that the addition of a further small amount of inactive formate does not make much difference in the percentage figures in Table VII. If this reasoning is correct, it can mean only that there is a

source of formate in the fasted rat liver other than the α-carbon of glycine. This explanation is in harmony with the results in Table V, which show an apparent decrease in the extent of the condensation of formate with glycine in nitrogen, as compared with that in air.

Inability of Carbon Dioxide to Form Formate—Table VIII shows that CO_2 does not go to formate, since the isolated formate and the β-carbon of serine have no counts. This experiment can also serve as a control experiment for the production of formate from glycine, since the C^{14} in the

TABLE VII

Dilution of C^{14} Concentration of β-Carbon of Serine by Added Inactive Formate

Atmosphere	Concentration of formate added	Per cent of total counts of serine in β-C
	mM per l	
Air	0.0	32, 37, 24
"	1.2	16
"	2.4	14
N	0.0	4
"	1.2	5
"	2.4	5, 5, 7

TABLE VIII

Inability of Carbon Dioxide to Form Formate

1 ml. of a $NaHCO_3$ solution (2.5 μM of CO_2) containing 669,000 counts per minute was added. Inactive glycine and inactive formate were introduced to give a concentration of 1.2 mM per liter each.

Experiment No.	Atmosphere	Recovered counts of serine				Glycine			Formate
		Total	α-C	β-C	Carboxyl C	Total	α-C	Carboxyl C	
1a	Air	0.28	0.00	0.00	0.32	0.05	0.00	0.05	0.00
1b	N	0.00				0.00			0.00

formate is determined by oxidizing the formate to CO_2 and collecting the CO_2.

Non-Reversibility of Glycine to Formate Reaction—Table V, taken together with Table IX, shows that labeled formate does not give rise to labeled glycine. In the experiments of Table IX glutamine was used as an ammonia source. (A glutaminase is present in liver and is active at the pH of the experiments (17).)

Reaction Mechanisms—The results reported here indicate that the conversion of glycine to serine is not a simple process. The over-all reaction apparently is represented by equations (2) and (3).

(2) $CH_2NH_2COOH + 2H_2O \rightarrow (CHOCOOH) \rightarrow HCOOH + CO_2 + NH_3 + 4H$

(3) $HCOOH + CH_2NH_2COOH + 2H \rightarrow CH_2OHCHNH_2COOH + H_2O$
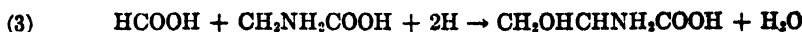
Though there is a glycine oxidase present in liver, it is not now known whether the metabolic pathway of glycine to serine operates through glyoxylic acid, though that seems the most logical intermediate at present. From equations (2) and (3), it appears that, while 1 glycine molecule is held seemingly immobile, the other is oxidized and partly converted to formate, the latter in turn condensing with the intact glycine. No other compound is known to the authors to be so metabolized. This is not a dismutation as written, in that 1 mole of glycine or glyoxylic acid is not reduced at the expense of another mole of glycine being oxidized. Also, the equations (2) and (3) show that there must be another hydrogen

TABLE IX

Inability of Bicarbonate to Condense with Formate in Presence of Glutamine to Form Glycine

1 ml. of radioactive sodium formate, equivalent to 0.6 mg. of formic acid, and containing 333,000 counts per minute, was added to make a final concentration of 1.2 mM per liter. The glutamine concentration was 1.2 mM per liter.

Experiment No.	Atmosphere	Recovered counts of serine		Glycine
		Total	β-C	
1a	N_2-CO_2	0.62	0.62	0.00
1b	O_2-CO_2	1.20	1.09	0.00

acceptor present in the system besides the reactants given in equation (3).

It might well be, as Bach has suggested (18), that glycine condenses with some other metabolite before being oxidized. This can explain the lack of oxidation of glycine when added to tissue slices, as found by him and by Siekevitz (7). It is evident that hydrogen acceptors must be present to explain the imbalance of the hydrogen atoms shown in equations (2) and (3).

Since we believe, for the reasons given above, that there is another source of formate in our system besides the α-carbon of glycine, the existence of this source may be a step towards the explanation of the discrepancy in the above equations. In experiments on the formation of uric acid, the isotopically labeled carboxyl group of acetic acid was found to occupy the same carbon positions as labeled formic acid (6). Experiments are now under way to determine whether acetate is the precursor of the extra glycine source of formate.

The authors are indebted to Dr. Theodore Winnick for valuable assistance and criticism in the conduct of the experimental work and in the preparation of the manuscript.

SUMMARY

1. The synthesis of serine from glycine by rat liver slices has been shown to occur both aerobically and anaerobically.

2. It has been demonstrated that labeled formate plus unlabeled glycine will give labeled serine, the radioactivity being all accounted for in the β-carbon.

3. The two carbon atoms of glycine behave differently in that the carboxyl carbon is metabolized probably to carbon dioxide, but definitely not to formate, while the α-carbon appears as formate. The production of formate from the α-carbon of glycine is not reversible under the conditions of our experiments.

4. Carbon dioxide is not reduced to formate by rat liver slices.

5. The formate formed from glycine condenses with another molecule of glycine to give serine. From the percentage of radioactivity in the β-carbon of serine, relative to the total radioactivity of the serine, it is concluded that there is another source of formate in the fasted rat liver slice, which is more active anaerobically than aerobically.

BIBLIOGRAPHY

1. Shemin, D., *J. Biol. Chem.*, **162**, 297 (1946).
2. Goldsworthy, P. D., Winnick, T., and Greenberg, D. M., *J. Biol. Chem.*, **180**, 341 (1949).
3. Winnick, T., Moring-Claesson, I., and Greenberg, D. M., *J. Biol. Chem.*, **175**, 127 (1948).
4. Siekevitz, P., Winnick, T., and Greenberg, D. M., *Federation Proc.*, **8**, 250 (1949).
5. Sakami, W., *J. Biol. Chem.*, **176**, 995 (1948).
6. Sonne, J. C., Buchanan, J. M., and Delluva, A. M., *J. Biol. Chem.*, **173**, 69 (1948).
7. Siekevitz, P., Thesis, University of California (1949).
8. Karlsson, J. L., and Barker, H. A., *J. Biol. Chem.*, **177**, 597 (1949).
9. Melchior, J. B., and Tarver, H., *Arch. Biochem.*, **12**, 301 (1947).
10. MacChesney, E. W., and Swann, W. K., *J. Am. Chem. Soc.*, **59**, 1116 (1937).
11. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, **150**, 251 (1943).
12. Rees, H. G., *Biochem. J.*, **40**, 632 (1946).
13. Herbst, R. M., and Shemin, D., *Org. Syntheses*, **19**, 4 (1939).
14. Osburn, O. L., Wood, H. G., and Werkman, C. H., *Ind. and Eng. Chem., Anal. Ed.*, **5**, 247 (1933).
15. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. F., Isotopic carbon, New York (1949).
16. Yankwich, P. F., Norris, T. H., and Huston, J. W., *Anal. Chem.*, **19**, 439 (1947).
17. Krebs, H. A., *Biochem. J.*, **29**, 1951 (1935).
18. Bach, S. J., *Biochem. J.*, **33**, 90 (1939).

SYNTHESIS AND SOME BIOLOGICAL PROPERTIES OF
4-AMINOPTEROYLASPARTIC ACID

By B. L. HUTCHINGS, J. H. MOWAT, J. J. OLESON, A. L. GAZZOLA, E. M.
BOGGIANO, D. R. SEEGER,* J. H. BOOTHE, C. W. WALLER, R. B.
ANGIER, J. SEMB, AND Y. SUBBAROW†

*(From the Lederle Laboratories Division, American Cyanamid Company, Pearl River,
New York)*

In an earlier communication the synthesis and certain of the anti-pteroylglutamic acid properties of pteroylaspartic acid were described (1). The related compound, N-[4-{[(2,4-diamino-6-pteridyl)methyl]amino}ben-zoyl]aspartic acid, which will be designated as 4-aminopteroylaspartic acid, has been prepared and the inhibitory nature of the compound on the growth of bacteria, chicks, and rats has been determined. By comparing the relative activities of the two compounds it is possible to assess the effect of the introduction of an amino group in place of the hydroxyl group on the pteridine ring on the antipteroylglutamic acid properties of this class of compounds.

EXPERIMENTAL

Synthesis of 4-Aminopteroylaspartic Acid

p-Aminobenzoylaspartic Acid—A mixture of *p*-nitrobenzoylaspartic acid (1) (640 gm.) and water (8 liters) was adjusted to pH 3.0 and reduced with zinc dust (800 gm.) and concentrated hydrochloric acid (1350 ml.) as described by Boothe *et al.* (2). When the reduction was complete, the excess zinc was removed by filtration and the filtrate was used in the following reaction.

4-Aminopteroylaspartic Acid—In a procedure similar to that described by Seeger *et al.* (3) for the synthesis of 4-aminopteroylglutamic acid, 2,4,5,6-tetraaminopyrimidine sulfate (4) (770 gm.) and 1,3,3-tribromo-propanone-2 (5) (430 ml.) were added to the well stirred filtrate containing the *p*-aminobenzoylaspartic acid. The mixture was then heated at 80° for 30 minutes and maintained at pH 2.0 by the addition of 2.5 N sodium hydroxide solution. The mixture was then adjusted to pH 3.5 to 4.0, cooled, and filtered. The filter cake was washed successively with 5 per cent sodium chloride solution, acetone, and ether.

---

* Present address, Calco Chemical Division, American Cyanamid Company, Bound Brook, New Jersey.
† Deceased, August 9, 1948.

This crude product was dissolved in 65 liters of 0.1 N sodium hydroxide solution at 35–40°, stirred for 30 minutes, and filtered. The filtrate was then heated to 70°, treated with 600 ml. of a 30 per cent calcium chloride solution, and again filtered. The filtrate was adjusted to pH 1.0 with hydrochloric acid, heated to 85–90°, and filtered while hot. On cooling, the product crystallized as small needles. Yield 131 gm. Chemical assay (6) indicated the product to be approximately 75 per cent pure. Further purification was effected by recrystallization from dilute hydrochloric acid.

$C_{19}H_{18}O_6N_8 \cdot HCl$. Calculated. C 46.71, H 4.14, N 24.21, Cl 7.66
                          Found.   " 46.69, " 4.6, " 24.52, " 7.39

Inhibition of Bacterial Growth—The effect of 4-aminopteroylaspartic acid on the growth of certain bacteria was investigated. The medium used for each organism was as follows: *Streptococcus faecalis* R was grown on the medium of Teply and Elvehjem (7), *Escherichia coli* BG-22[1] was cultured on a similar medium adjusted to pH 6.0 from which the purines and pyrimidines were omitted, and *Lactobacillus arabinosus* was grown on a modified Teply and Elvehjem medium in which sodium acetate was used as the buffer and only 1 per cent glucose was added; *L. arabinosus* and *E. coli* were incubated 16 hours at 37° and *S. faecalis* R was incubated 16 hours at 32°.

When the inhibition index is reported, it is defined as the ratio of inhibitor to metabolite that will produce one-half maximum inhibition.

Rat and Chick Experiments—The experimental conditions were similar to those previously described (1).

Results

Bacterial Inhibition—The inhibition indices for a number of the pteroyl compounds as metabolites of *S. faecalis* R are tabulated in Table I. With *S. faecalis* R, 4-aminopteroylaspartic acid is a typical competitive antagonist for pteroylglutamic acid and related compounds. The action of the inhibitor is similar to that of pteroylaspartic acid but different from that of 4-aminopteroylglutamic acid. Oleson *et al.* (8) showed the latter compound to be a strong inhibitor of the growth of *S. faecalis* R. The inhibition could be reversed by the addition of excess pteroylglutamic acid, but it was noted that the reversal of the inhibition by the addition of graded amounts of pteroylglutamic acid was not a direct function of the ratios of the concentration of metabolite to inhibitor. The present data indicate that the replacement of the hydroxyl group of the pteridine ring by an amino group is not the sole determining factor for the abnormal behavior of 4-aminopteroylglutamic acid.

[1] This culture was originally isolated in these laboratories in 1917.

It is interesting to note that 4-aminopteroylaspartic acid is considerably more active than pteroylaspartic acid in inhibiting the growth induced by pteroic acid or pteroylglutamic acid. When the metabolites pteroyl-γ-glutamylglutamic acid and pteroyl-γ-glutamyl-γ-glutamylglutamic acid are used, the two antagonists show approximately the same activity. This is most probably due to the fact that the pteroyltriglutamic acid compound is utilized with difficulty by the organism. Hence, the association between the metabolite and enzyme is so weak that this, rather than the inhibitory nature of the antagonist, becomes the limiting

TABLE I

4-Aminopteroylaspartic Acid Inhibition of Metabolites for Streptococcus faecalis R

Metabolite	Concentration of metabolite	Inhibition index*
	γ per 10 ml.	
Pteroic acid	0.004	0.35
" "	0.04	0.39
" "	0.4	0.15
Pteroylglutamic acid	0.003	1.65
" "	0.03	0.50
" " . .. .·	0.3	0.23
" "	3.0	0.73
Pteroyl-γ-glutamylglutamic acid	0.005	4.12
" "	0.05	2.5
" "	0.5	1.79
Pteroyl-γ-glutamyl-γ-glutamylglutamic acid	0.07	1.0
" "	0.7	0.28
" "	7.0	0.15

* Ratio of the amount of inhibitor to metabolite that will produce half maximum inhibition.

factor. The pteroyldiglutamic acid compound would also fall in this category.

The effect of 4-aminopteroylaspartic acid on the growth of *S. faecalis* R, *E. coli*, and *L. arabinosus* is outlined in Table II. *E. coli* and *L. arabinosus*, as examples of bacteria that do not require preformed pteroylglutamic acid, were completely inhibited by the addition of from 300 to 500 γ and 10 to 15 γ of 4-aminopteroylaspartic acid per 10 ml. of medium respectively. Data are presented on the results of the addition of compounds that have been shown to replace pteroylglutamic acid in the nutrition of various lactic acid bacteria (9, 10) or that have been shown to function in the synthesis of thymidine in certain microorganisms (11, 12).

<div align="center">

TABLE II

Effect of Various Compounds on 4-Aminopteroylaspartic Acid Inhibition

</div>

When negative results are tabulated, the figure in parentheses represents the highest concentration used. When positive results are expressed, the effective concentration of the compound is indicated in parentheses. ± indicates half maximum reversal.

Organism	Compounds			
	Pteroylglutamic acid	Thymine	Vitamin B$_{12}$	Thymidine*
?. arabinosus	− (1 mg.)	− (4 mg.)	− (10 γ)	± (0.1 mg.)
?. coli...	− (1 ")	± (0.5 mg.)	− (10 ")	± (0.1 ")
?. faecalis R.	+·	+ (7.5 γ)	− (10 ")	+ (0.1 ")

* Used as a concentrate.

<div align="center">

TABLE III

Effect of 4-Aminopteroylaspartic Acid on Chicks

</div>

Group No.	Supplement	Average weights*			
		Initial	7 days	14 days	21 days
		gm	gm.	gm.	gm.
1	None	39 (7)	76 (7)	105 (7)	110 (5)
2	0.25 mg. pteroylglutamic acid per kilo diet	38 (7)	83 (7)	147 (7)	212 (7)
3	As (2) + 0.25 mg. 4-aminopteroylaspartic acid per kilo diet	39 (7)	71 (7)	134 (6)	202 (6)
4	As (2) + 1.0 mg. 4-aminopteroylaspartic acid per kilo diet	39 (7)	76 (6)	134 (5)	196 (5)
5	As (2) + 2.0 mg. 4-aminopteroylaspartic acid per kilo diet	39 (7)	71 (7)	135 (7)	202 (7)
6	As (2) + 4.0 mg. 4-aminopteroylaspartic acid per kilo	39 (7)	74 (7)	130 (7)	185 (7)
7	None	39 (8)	60 (8)	72 (7)	
8	As (7) + 25 γ pteroylglutamic acid†	39 (8)	71 (8)	133 (8)	
9	" (7) + 0.5 mg. 4-aminopteroylaspartic acid†	39 (8)			
10	As (9) + 5 γ pteroylglutamic acid†	39 (8)	51 (5)	95 (3)	
11	" (9) + 10 γ " " †	39 (8)	51 (7)	106 (6)	
12	" (9) + 15 γ " " †	39 (8)	59 (7)	111 (7)	
13	" (9) + 20 γ " " †	38 (8)	64 (7)	114 (8)	
14	None	46 (8)	75 (8)	99 (7)	
15	As (14) + 50 γ pteroylglutamic acid†	47 (8)	84 (8)	140 (8)	
16	" (14) + 0.5 mg. 4-aminopteroylaspartic acid†	46 (8)	55 (6)	76 (1)	
17	As (16) + 20 γ pteroylglutamic acid†	46 (8)	73 (8)	128 (8)	
18	" (16) + 40 " " " †	47 (8)	79 (6)	127 (6)	
19	" (16) + 50 " " " †	47 (8)	87 (7)	139 (7)	

* The figures in parentheses indicate the number of survivors.

† Intramuscularly five times weekly.

With *S. faecalis* R the growth inhibition induced by 4-aminopteroyl-aspartic acid was completely reversed by the addition of pteroylglutamic acid, thymine, or a thymidine concentrate. However, the growth result-ing from the addition of thymine or thymidine could not be inhibited by extremely high levels of 4-aminopteroylaspartic acid (2 mg. per 10 ml.).

Thymine and thymidine were able to reverse the inhibition partially when *E. coli* was used as the test organism. With *L. arabinosus* only the

TABLE IV

Effect of 4-Aminopteroylaspartic Acid on Rat

Group No.		Average weights*				
		Initial	1 wk.	2 wks.	3 wks.	4 wks.
1	None	39 (7)	56 (7)	82 (7)	102 (7)	144 (7)
2	3.0 mg. 4-aminopteroylaspartic acid†	39 (10)	56 (10)	81 (10)	112 (10)	144 (10)
3	6.0 mg. 4-aminopteroylaspartic acid†	42 (9)	46 (9)	77 (5)	109 (5)	136 (5)
4	As (3) + 60 γ pteroylglutamic acid†	40 (4)	58 (4)	82 (4)	111 (4)	142 (4)
5	None	41 (7)	50 (7)	74 (7)	104 (7)	129 (7)
6	0.5 mg. 4-aminopteroylaspartic acid‡	43 (5)	51 (5)	71 (5)	92 (5)	116 (5)
7	1.0 mg. 4-aminopteroylaspartic acid‡	43 (5)	50 (5)	63 (5)	86 (5)	109 (5)
8	3.0 mg. 4-aminopteroylaspartic acid‡	41 (10)	46 (10)	60 (8)	80 (5)	101 (5)
9	None	44 (8)	63 (8)	91 (8)	120 (8)	146 (8)
10	0.5 mg. 4-aminopteroylaspartic acid‡	45 (8)	58 (8)	78 (6)	103 (6)	133 (5)
11	As (10) + 50 γ pteroylglutamic acid‡	45 (8)	59 (8)	72 (8)	105 (7)	132 (7)
12	As (10) + 100 γ pteroylglutamic acid‡	44 (6)	61 (6)	89 (6)	117 (6)	147 (6)

* The figures in parentheses indicate the number of survivors.
† Orally five times weekly.
‡ Intramuscularly five times weekly.

thymidine concentrate had a partial effect. The inhibition of this organ-ism appears to be due in part to an interference in the synthesis of thy-midine. These results are similar to those noted by Shive and coworkers (13) for *Leuconostoc mesenteroides*.

Various combinations of the compounds were tried, but no combination was more effective than the single compounds noted.

Chick—The results of the various chick experiments are tabulated in Table III. The data indicate that the route of administration is important. When administered orally, the compound is inactive at a ratio of metabolite to inhibitor of 1:16. On intramuscular injection the inhibitor is markedly more effective. At a ratio of 1:10 the growth effects of pteroylglutamic acid are neutralized. It is apparent with chicks and bacteria that 4-aminopteroylaspartic acid is approximately 50 times as active as pteroylaspartic acid in reversing the growth effects of pteroylglutamic acid.

Rat—As an inhibitor of the growth of rats 4-aminopteroylaspartic acid is markedly inferior to the corresponding glutamic acid analogue. The oral feeding of 6 mg. of the inhibitor per day produces approximately 50 per cent mortality and a partial inhibition of growth in the survivors. When 3 mg. per day are injected intramuscularly, a more pronounced growth inhibition is evident. With 0.5 mg. intramuscularly some growth retardation occurs. This effect is counteracted by the injection of 100 γ of pteroylglutamic acid. The inhibitor produced no gross symptoms of pteroylglutamic acid deficiency other than that of a general retardation in growth.

DISCUSSION

Under the conditions of the present investigation, 4-aminopteroylaspartic acid behaves as a competitive metabolite antagonist in that the degree of inhibition is a function of the concentration of the metabolite.

The deficiency induced in the chick and the rat is not of a precipitous nature but is characterized by a gradual onset of the deficiency symptoms more akin to those of a simple dietary depletion of the vitamin. These results are in contrast to those obtained previously with 4-aminopteroylglutamic acid. It is of interest to speculate whether the 4-amino group is responsible for the strong inhibitory action of the glutamic acid analogue or whether the activity of the compound can be presumed to be due to its very close structural similarity to pteroylglutamic acid. If the amino group is responsible, other 4-amino analogues of the pteroyl compounds should also show to a certain extent the biological properties associated with 4-aminopteroylglutamic acid. The present results would indicate that the 4-amino group increases the inhibitory power of the compound but that the competitive nature of the antagonist is retained.

The enhanced activity of 4-aminopteroylaspartic acid is reflected in the increased inhibitory activity of the compound for bacteria and the chick but more noticeably in the ability of the 4-amino derivative to inhibit the growth of bacteria that do not require preformed pteroyl-

glutamic acid and to produce a growth retardation in the rat. Pteroylaspartic acid was inactive under these latter conditions.

SUMMARY

The synthesis of 4-aminopteroylaspartic acid from *p*-aminobenzoylaspartic acid, 1,3,3-tribromopropanone-2, and 2,4,5,6-tetraaminopyrimidine is described. The compound is a competitive antagonist of pteroylglutamic acid in the chick, the rat, and in certain bacteria that require preformed pteroylglutamic acid as a metabolite.

It is a pleasure to express our appreciation to Mr. L. Brancone and coworkers for the microanalyses.

BIBLIOGRAPHY

1. Hutchings, B. L., Mowat, J. H., Oleson, J. J., Stokstad, E. L. R., Boothe, J. H.,
   . Waller, C. W., Angier, R. B., Semb, J., and SubbaRow, Y., *J. Biol. Chem.*, **170,** 323 (1947).
2. Boothe, J. H., Semb, J., Waller, C. W., Angier, R. B., Mowat, J. H., Hutchings, B. L., Stokstad, E. L. R., and SubbaRow, Y., *J. Am. Chem. Soc.*, **71, 2304** (1949).
3. Seeger, D. R., Cosulich, D. B., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, **71,** 1753 (1949).
4. Traube, W., *Ber. chem. Ges.*, **37,** 4545 (1904).
5. Watson, H. B., and Yates, E. D., *J. Chem. Soc.*, 1207 (1932).
6. Hutchings, B. L., Stokstad, E. L. R., Boothe, J. H., Mowat, J. H., Waller, C. W., Angier, R. B., Semb, J., and SubbaRow, Y., *J. Biol. Chem.*, **168, 705** (1947).
7. Teply, L. J., and Elvehjem, C. A., *J. Biol. Chem.*, **157, 303** (1945).
8. Oleson, J. J., Hutchings, B. L., and SubbaRow, Y., *J. Biol. Chem.*, **175, 359** (1948).
9. Stokstad, E. L. R., *J. Biol. Chem.*, **139,** 475 (1941).
10. Stokes, J. L., *J. Bact.*, **48,** 201 (1944).
11. Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, **70, 2614** (1948).
12. Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, **176,** 1459 (1948).
13. Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.*, **70,** 2299 (1948).

THE PRIMARY AND SECONDARY COMPOUNDS OF CATALASE AND METHYL OR ETHYL HYDROGEN PEROXIDE*

III. REACTIONS IN CONCENTRATED ALKYL HYDROGEN PEROXIDE SOLUTIONS

By BRITTON CHANCE†

(From the Biochemical Department of the Nobel Medical Institute, Stockholm, Sweden)

The experiments in Paper II (1) have indicated that the velocity of the spontaneous decomposition of the primary and secondary catalase alkyl hydrogen peroxides is very slow in dilute alkyl hydrogen peroxide solutions ($k_3 \sim 2 \times 10^{-2}$ and 1×10^{-4} sec.$^{-1}$ respectively). In concentrated ethyl hydrogen peroxide solutions, the molarity of alcohol present in the alkyl hydrogen peroxide solutions could be sufficient to cause appreciable peroxidatic activity of complex I. However, catalase is largely in the form of complex II under these conditions, and the latter has negligible peroxidatic activity ($k_4 \sim 1 \times 10^{-2}$ M^{-1} $\times$ sec.$^{-1}$) compared with the primary complex ($\sim 1 \times 10^{3}$ M^{-1} $\times$ sec.$^{-1}$).

Stern (2) has, however, made spectroscopic and titrimetric studies of the decomposition of ethyl hydrogen peroxide by catalase in relatively concentrated catalase and ethyl hydrogen peroxide solutions and has concluded that the red secondary catalase-ethyl hydrogen peroxide complex discovered by him has the properties of a Michaelis intermediate in this reaction. Complex I was not, however, recognized by Stern at that time.

In this paper, calculations based upon Stern's data and new experimental data indicate that the secondary catalase-ethyl hydrogen peroxide complex is not responsible for the observed decomposition of ethyl hydrogen peroxide according to the Michaelis theory. A small amount of the primary compound has been detected in this reaction and affords a partial explanation of the disappearance of ethyl hydrogen peroxide according to the Michaelis theory.

Calculation of Activity of Catalase in Concentrated Ethyl Hydrogen Peroxide Solutions on Basis of Stern's Data—Assuming that complex II is the Michaelis intermediate in this reaction, its rate of breakdown (k_3) or the turnover number can be easily calculated from the Michaelis constant (K_m) by the simple relation $K_m = (k_3 + k_2)/k_1$ (see (1), Equations 3 and 4) where k_1 is the rate of formation and k_2 the rate of reversible break-

down of complex II. According to Stern's paper, the equations would be written

$$\text{Cat} + \text{C}_2\text{H}_5\text{OOH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Cat}\cdot\text{C}_2\text{H}_5\text{OOH}$$

$$\text{Cat}\cdot\text{C}_2\text{H}_5\text{OOH} \overset{k_3}{\longrightarrow} \text{Cat} + \text{reaction products}$$
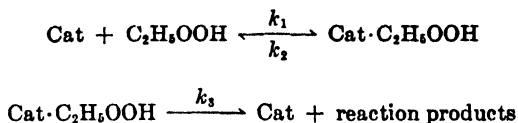
Stern (2) has already given spectroscopic and activity data for K_m. According to his spectroscopic data, the ethyl hydrogen peroxide concentration giving complete conversion of catalase to complex II is 0.3 M, and the value for half conversion (K_m) is no greater than 0.1 M because of the hyperbolic nature of a saturation characteristic and because of the disappearance of ethyl hydrogen peroxide during the formation of complex II. On the other hand, Stern's activity-substrate relationship gives K_m = 0.02 M. Thus K_m lies between 0.02 and 0.1 M. Stern and DuBois (3) have shown that complex II is half formed in 2 seconds (see Chance (1)) in 0.1 M ethyl hydrogen peroxide. Thus $k_1 = 3.5$ M^{-1} × sec.$^{-1}$. Assuming k_2 = 0, the value of k_3 is $k_3 = k_1 K_m$, or k_3 lies between 0.35 and 0.07 sec.$^{-1}$. If $k_2 \neq 0$, k_3 is even less than these values.

It can now be determined whether the turnover of ethyl hydrogen peroxide based upon the titrimetric data of Stern's Table II (2) is consistent with this value of k_3.

At 23° the reaction is apparently of zero order, as might be expected when the initial substrate concentration is 0.2 M and the Michaelis constant is 0.02 M (at 10°); at least the reaction should be of zero order in its initial phases. The fact that the reaction is zero order when the substrate concentration falls to 0.006 M is an inconsistency not discussed by Stern.

Assuming, however, that the enzyme is saturated with substrate, the rate of breakdown of the enzyme-substrate compound is given by the equation, $k_3 = (dx/dt \times 1/e)$ (see (1), Equation 9). dx/dt is the rate of disappearance of ethyl hydrogen peroxide and e is the catalase concentration. dx/dt is about 0.8 cc. of 0.1 N C$_2$H$_5$OOH per minute or $(8 \times 10^{-4} \times 0.1)/(2 \times 60) = 6.7 \times 10^{-7}$ mole per second in a total volume of 5 cc. or, on a molar basis, 1.3×10^{-4} M × sec.$^{-1}$.

The concentration of the stock catalase solution is obtained on the assumption that Stern's catalase preparation would have had $Kat f = 30,000$ had he purified it. From the definition of $Kat f$, the gm. of catalase per 50 cc. = $2020/30,000 = 6.7 \times 10^{-2}$. If gm. per 50 cc. are converted to moles per liter, $(6.7 \times 10^{-2} \times 10^3)/(2.25 \times 10^5 \times 50) = 6 \times 10^{-6}$ M. But since this solution was diluted at 2/5, $e = 2.4 \times 10^{-6}$ M catalase or 7.2×10^{-6} M hematin iron and $k_3 = dx/dt \times 1/e = (1.3 \times 10^{-4})/(7.2 \times 10^{-6}) = 18$ sec.$^{-1}$ from the data of Table II (2). From Stern's Fig. 4 (2), k_3 is cal-

culated to be 34 sec.$^{-1}$ in 0.1 M ethyl hydrogen peroxide and 6 sec.$^{-1}$ in 0.01 M ethyl hydrogen peroxide at 23°. All these values are large compared to k_3 calculated from K_m above.

At 0° the kinetic data are not consistent with those at 23°, and the reaction, strangely enough, is quite accurately of the first order in spite of the fact that the Michaelis constant at this temperature, 0.02 M, is considerably less than the initial substrate concentration. The activity may be calculated as for catalase and hydrogen peroxide; on the assumption that the free enzyme or a saturated intermediate reacts with the substrate

$$2.3 \log_{10} \frac{x_0}{x} = k_1 e t$$
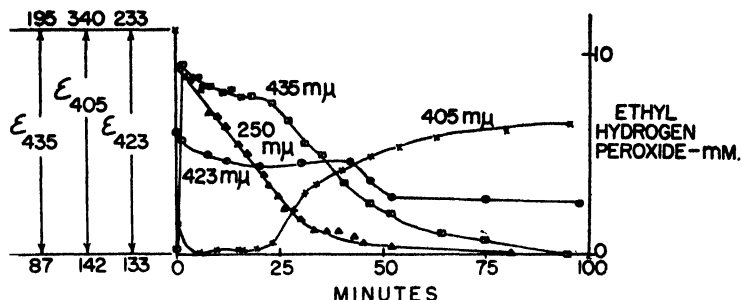
FIG. 1. The kinetics of catalase ethyl hydrogen peroxide compound I (423 mμ) and compound II (435 mμ) and compound I + II (405 mμ) and of the disappearance of ethyl hydrogen peroxide (250 mμ) measured in the Beckman spectrophotometer. The extinction coefficients (ϵ) of free catalase at 405, 423, and 435 mμ are indicated by the first point on the curves at $t = 0$. The units of ϵ are cm.$^{-1}$ × mM^{-1}. 3.5 μM of hematin iron horse liver catalase; 9.5 mM of initial ethyl hydrogen peroxide; pH 6.5; 0.01 M phosphate; temperature 25° (Experiment 148).

where k_1 is the rate of reaction of enzyme and substrate and t is in seconds. k_3 is not calculated in this case, since the first order kinetics indicate that k_3 is not a rate-determining step under these conditions. The quantity k_1 can be readily computed from Stern's Table II, $(1/t) \log_{10} (x_0/x) = 0.022$ min.$^{-1}$, and on substitution

$$\frac{2.3 \times 0.022}{60 \times 10.7 \times 10^{-6}} = 80 \text{ M}^{-1} \times \text{sec.}^{-1}$$

or, at 20°, about 400 M^{-1} × sec.$^{-1}$.

On the basis of Stern's spectroscopic and kinetic data $k_3 \leq 0.35$ sec.$^{-1}$, and, on the basis of his titrimetric data, a value of about 34 sec.$^{-1}$ is required in 0.1 M ethyl hydrogen peroxide; the discrepancy is in excess of 100-fold. A discrepancy exists if the values of k_1 from spectroscopic (3.5

M^{-1} × sec.$^{-1}$) and titrimetric (400 M^{-1} × sec.$^{-1}$) tests are compared, again in excess of 100-fold. The inconsistencies in Stern's spectroscopic and titrimetric data are obvious.

Simultaneous Measurements of Kinetics of Ethyl Hydrogen Peroxide and Catalase—We have repeated Stern's experiments in a somewhat more comprehensive fashion, and the results are shown in Figs. 1 and 2.

It is the purpose of these experiments to determine whether the green primary complex I or the red secondary complex II of catalase and ethyl hydrogen peroxide is the Michaelis enzyme-substrate complex in this reaction. By observing both the over-all reaction and the enzyme-substrate kinetics the definitive correlations developed in the study of peroxidase can be applied here.

No difficulty has been encountered in obtaining approximately simultaneous measurements of the kinetics of the disappearance of ethyl hydro-
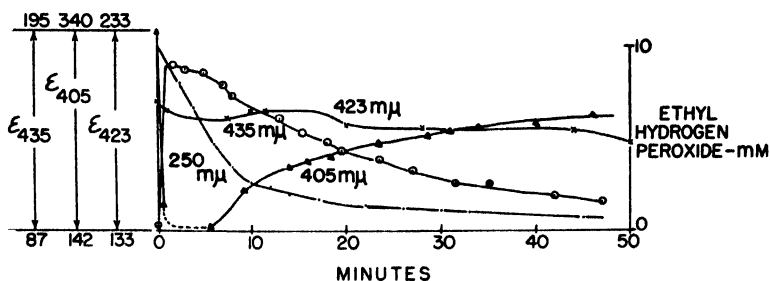
Fig. 2. The acceleration of the disappearance of ethyl hydrogen peroxide in the presence of 10 mM of ethanol. Other conditions are the same as in Fig. 1.

gen peroxide and the shifts in the catalase spectrum at three wave-lengths from a single experiment with the ordinary Beckman spectrophotometer without any special mixing devices. The disappearance of ethyl hydrogen peroxide was measured at the minimum of the catalase band at 250 mμ where the extinction coefficient of ethyl hydrogen peroxide is 0.0132 cm.$^{-1}$ × mM^{-1} (Reiche (4)). The assumption was made that no shift of the catalase band occurs at this wave-length during the reaction, an assumption which is supported by the absence of any shift caused by other substances, for example, cyanide. However, the total density change during the reaction was about 25 per cent less than that expected, owing possibly to the absorption of an oxidation product of ethyl hydrogen peroxide.

The wave-lengths selected for the study of catalase are 423 mμ, at which complex I alone is registered, 435 mμ, at which complex II alone is registered, and 405 mμ, at which both complexes I and II are registered (5). Ethyl hydrogen peroxide has negligible absorption at these wave-lengths.

In Fig. 1 the disappearance of ethyl hydrogen peroxide is seen to follow a zero order course as found by Stern at 23°. If k_3 ($k_3 = dx/dt \times 1/e$) is calculated for a saturated enzyme-substrate complex in which all three horse liver catalase hematins are involved, a value of 1.4 sec.$^{-1}$ is found, which is somewhat smaller than the value of 6 sec.$^{-1}$ calculated from Stern's data for 0.01 M ethyl hydrogen peroxide.
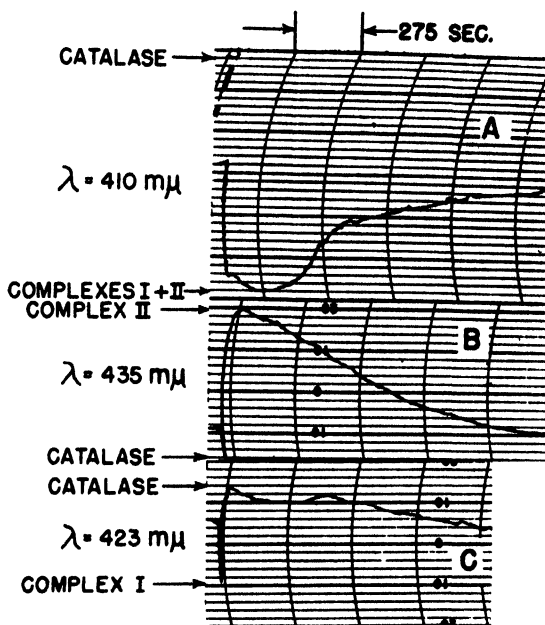
FIG. 3. Recordings of the kinetics of catalase ethyl hydrogen peroxide I and II obtained in the capillary of the rapid flow apparatus. The initial phases of the reactions of Figs. 1 and 2 are more clearly shown here. 3.4 μM of hematin iron horse liver catalase; 10 mM of ethanol; 10 mM of ethyl hydrogen peroxide; temperature 28° (Experiment 147).

At 435 mμ, the kinetics of catalase in Figs. 1 and 2 demonstrate the gross inconsistency between the concentration of complex II and the rate of reaction measured at 250 mμ; the concentration of complex II remains very near its saturation value, even though the rate of disappearance of the ethyl hydrogen peroxide has fallen to a very small value. This indicates that complex II is not a Michaelis intermediate in this reaction. In quantitative terms, a Michaelis intermediate cannot exist at its saturation value if its rate of breakdown (1.4 sec.$^{-1}$) is 14 times greater than its rate of formation (see Chance (1), Table III). At 405 mμ, the sum of compounds I and II is registered and is seen to remain at a saturation value

as long as the reaction is of zero order. At the end of the reaction, only 60 per cent of the initial catalase concentration is recovered. Possible explanations are that there is still sufficient ethyl hydrogen peroxide present to give an appreciable concentration of complex I or that some of the enzyme has been destroyed. At 423 mμ, the concentration of complex I is registered, except that its very rapid initial formation and nearly complete disappearance are not shown, since this occurred in the first 10 seconds of the reaction (see Fig. 3). The kinetics do show that a measurable amount of complex I exists during the reaction.

In the presence of added ethanol (10 mM), the disappearance of ethyl hydrogen peroxide is accelerated, as shown in Fig. 2, and $k_3' = dx/dt \times 1/e = 4.8$ sec.$^{-1}$, calculated on the basis of a saturated enzyme-substrate complex. The kinetics of catalase are similar to those of Fig. 1, except that the correspondence between the zero order disappearance of ethyl hydrogen peroxide and the concentration of complexes I and II registered at 405 mμ is much better in Fig. 2 than in Fig. 1. Again an appreciable concentration of complex I is seen at 423 mμ. The inconsistency between the kinetics of complex II recorded at 435 mμ and the over-all reaction is apparent.

In order to demonstrate that there is a small concentration of complex I in this reaction, the same experiment was carried out in the rapid flow apparatus and the kinetics at these three wave-lengths are shown by Fig. 3. The difference between the kinetics at 435 and 410 mμ (the kinetics at 410 mμ differ but little from those at 405 mμ; see Chance (5)) is very distinct, but the kinetics at 410 mμ show a definite similarity to those at 423 mμ in the light of the following interpretation.

The observation tube of the rapid flow apparatus is initially filled with some complex II formed in a previous identical experiment. Initiation of the flow of reactants does not give the optical density of free catalase; complex I forms in a half time of about 7 milliseconds. The records therefore show the partial formation of complex I at the fastest flow velocities at both 410 and 423 mμ. When the flow stops (the flow lasts for about 0.3 second), complex I then changes into complex II to an extent depending upon the catalase, ethyl hydrogen peroxide, and ethanol concentrations. This change is clearly shown by the rapid upward deflection of the tracing at 423 mμ. Under these conditions, a small steady state concentration of complex I still exists, as illustrated by the definite downward deflection at 423 mμ with respect to the point marked "catalase." As the ethyl hydrogen peroxide disappears, the concentration of complex I increases somewhat, as shown by the increasing downward deflections at both 410 and 423 mμ. At the same time, the concentration of complex II decreases, owing to the conversion of complex II to I. However, the kinetics of com-

plex II cannot follow those of complex I instantaneously because of the slower breakdown of complex II. Thus the relatively rapid upward deflection at 410 mμ at about 300 seconds is caused by a decrease of the concentration of complex I and the release of some free catalase when the bulk of the ethyl hydrogen peroxide is exhausted. There is a less definite but corresponding change at 423 mμ at 360 seconds. After this time, the slow conversion of complex II to I and to free catalase continues, which gives only a small differential change at 410 mμ.

Based upon this interpretation of these curves, the amount of complex I after the reaction has proceeded for several minutes is estimated at 20 per cent directly from the curve at 423 mμ. This amount of complex I causes the peroxidatic activity measured at 250 mμ in Fig. 2. The rate of breakdown (k_3) calculated in Fig. 2 for a saturated enzyme-substrate complex can now be corrected for the fact that only 20 per cent of the catalase is in the form of complex I. Thus $k_3' = 4.8/0.20 = 24$ sec.$^{-1}$.

It is of considerable interest to determine whether the value of k_4 for the reaction of complex I with ethanol is the same in this reaction as that found previously (1). Equation 12 (see Chance (1)) is used for this calculation. If k_3 is taken to be 5.6 sec.$^{-1}$ (see below) and k_3' as 24 sec.$^{-1}$, $k_3' = k_3 + k_4 a_0$, $k_4 = (24 - 5.6)/0.01 = 1800$ M$^{-1} \times$ sec.$^{-1}$. There is rough agreement with the value of k_4 found in more dilute ethyl hydrogen peroxide solutions (2100 M$^{-1} \times$ sec.$^{-1}$) (1). Thus the amount of complex I present in Figs. 2 and 3 is adequate to account for the disappearance of ethyl hydrogen peroxide by the ordinary peroxidatic mechanism.

In order to explain the disappearance of ethyl hydrogen peroxide in Fig. 1, an estimate of the concentration of complex I is required. This is more difficult to estimate in Fig. 1 than in Figs. 2 and 3, because the kinetics of complex I are not so clearly distinguished from those of complex II. Roughly estimated from the extinction at 423 mμ, the concentration of complex I is about 25 per cent. The value of k_3' calculated on the basis of 25 per cent of the catalase in the form of complex I is $1.4/0.25 = 5.6$ sec.$^{-1}$. Equation 13 (1) can now be used to compute the amount of ethanol which is required to raise k_3 to 5.6 sec.$^{-1}$. This is 2.5 mM of ethanol. On the basis of 10 mM of initial ethyl hydrogen peroxide, this represents 25 per cent and is considerably larger than the value previously obtained (on the basis of the variation of k_3', with x_0 in Table V (1) f was 0.08, or only 8 per cent of the ethyl hydrogen peroxide solution could be ethanol).

Whereas the reaction of complex I with ethanol affords a satisfactory explanation of Figs. 2 and 3, not enough ethanol is present in Fig. 1 to provide a complete explanation. Only 30 per cent of the activity can be accounted for in this manner. Nevertheless, the agreement is very much closer than in the calculations above, which were based on the kinetics

of complex II in which only 1 per cent of the activity can be explained. Two suggestions for accounting for the discrepancy of the calculation based on complex I are given below.

Direct evidence for the presence of some acceptor (probably ethanol) was obtained by a redistillation of the stock ethyl hydrogen peroxide solution after dilution with an equal volume of water. Two fractions, one-third the total volume each, were taken, and the activity of catalase in the presence of equal concentrations of ethyl hydrogen peroxide in these two fractions was measured under the same conditions as in Fig. 1 (ethyl hydrogen peroxide concentration 9 mM). The values of k_3' were found to be 0.92 and 0.46 sec.$^{-1}$ for the first and second distillates respectively, as could be expected from the relatively higher volatility of ethanol.

It is very important to note that the initial ethanol concentration may not be depleted in this reaction because ethanol is a reduction product of ethyl hydrogen peroxide (6).

The measured concentration of complex I, its known reactivity towards ethanol, and the indication that ethanol is present in the ethyl hydrogen peroxide solution partially account for the velocity of decomposition of strong ethyl hydrogen peroxide by catalase. The slow rate of formation of complex II and its existence at its saturation value during the decomposition of ethyl hydrogen peroxide are incompatible with the observed rate of decomposition of ethyl hydrogen peroxide. According to the Michaelis theory, complex II can make a negligible contribution to the activity. However, in order to explain the high values of k_3 in Fig. 1 and in Stern's tests, the following suggestions may be made. (1) The spontaneous breakdown of complex I (k_3) is larger in strong ethyl hydrogen peroxide solutions. (2) In strong ethyl hydrogen peroxide solutions, the catalase solutions employed may exert a catalytic influence not related to the concentration of complex I or II (iron itself is a catalyst in the decomposition of diethyl peroxide (7)).

Activity of Catalase towards Strong Methyl Hydrogen Peroxide—By using the same technique as in Figs. 1 and 2, the disappearance of 11 mM of methyl hydrogen peroxide in the presence of 3.9 μM of hematin iron horse blood catalase was measured. The values of k_3 computed on the basis of the total enzyme concentration (not upon the amount in the form of the primary compound) are 1.8 sec.$^{-1}$ and, with the addition of 10 mM of ethanol, 2.0 sec.$^{-1}$. Thus the decomposition of strong methyl hydrogen peroxide proceeds at about the same rate as that of strong ethyl hydrogen peroxide. The much smaller increment of rate upon the addition of ethanol may be caused by the more complete conversion of complex I to complex II in the presence of strong methyl hydrogen peroxide.

SUMMARY

In strong ethyl hydrogen peroxide solutions, the rapid disappearance of ethyl hydrogen peroxide, the slow formation of catalase ethyl hydrogen peroxide II, and the large concentration of this complex are incompatible with Stern's conclusion (2) that this compound has the properties postulated by Michaelis and Menten for an enzyme-substrate compound. Under these conditions, the presence of complex I has been demonstrated and, owing to ethanol present in the ethyl hydrogen peroxide solutions, can account for a portion of the observed activity by means of the peroxidatic reaction found previously (1). On addition of a known amount of ethanol, the increment in the rate of disappearance of ethyl hydrogen peroxide is roughly equal to that expected from measurements of the peroxidatic activity of complex I in dilute solutions.

BIBLIOGRAPHY

1. Chance, B., *J. Biol. Chem.*, **179,** 1341 (1949).
2. Stern, K. G., *J. Biol. Chem.*, **114,** 473 (1936).
3. Stern, K. G., and DuBois, D., *J. Biol. Chem.*, **116,** 575 (1936).
4. Reiche, A., Alkylperoxide und Ozonide, Erlangen, **62,** 111 (1930).
5. Chance, B., *J. Biol. Chem.*, **179,** 1331 (1949).
6. Chance, B., *J. Biol. Chem.*, in press.
7. Wieland, H., and Chrometzka, F , *Ber. chem. Ges.*, **63,** 1028 (1930).

ENZYMATIC SYNTHESIS OF A COMPOUND WITH ACETYL-CHOLINE-LIKE BIOLOGICAL ACTIVITY*

By DAVID NACHMANSOHN, SHLOMO HESTRIN,† AND
HELEN VORIPAIEFF

(From the Department of Neurology, College of Physicians and Surgeons,
Columbia University, New York)

Previous work on the enzymatic acetylation of choline required the use of bioassays (1, 2). The concentrations of acetylcholine formed were small. No adequate chemical method for the determination of the ester was available. These difficulties have been overcome.

The choline-acetylating system of rabbit brain has been purified and concentrated by fractional ammonium sulfate precipitation. The procedure was as follows: Acetone-dried powder or rabbit brain is extracted at 4–6°, with 15 ml. of a solution, per gm. of powder, of the following composition: KCl 0.2 M; $MgCl_2$ 1 mM; Na_2HPO_4 6 mM; NaCl 2 mM. The pH is maintained at 7.2. The suspension is centrifuged and the supernatant is fractionated with ammonium sulfate solution. The precipitate formed at 16 per cent ammonium sulfate is inactive and may be discarded. The precipitate obtained when the concentration of ammonium sulfate is increased to 36 per cent contains the bulk of the activity. The precipitate is taken up in about 2 ml. of a solution of the composition described above, supplemented with cysteine 2 mM and dialyzed for 3 hours against the same solution in the cold (4–6°). The enzyme solution keeps at 4° for several days without loss of activity.

The test system contained the following (micromoles per ml.): choline chloride 10, sodium acetate 10, adenosine triphosphate 6, cysteine 30, KCl 40, $MgCl_2$ 0.7, $CaCl_2$ 2, sodium phosphate 1.5; pH 7.0 to 7.2. The added acetate was found to be an essential component. Coenzyme was added in the form of a soluble dry preparation from hog liver at a final concentration of 2 mg. of powder per ml.[1] 1 ml. of reaction mixture usually contained 0.15 ml. of enzyme solution. To inactivate any cholinesterase which might still be present, tetraethyl pyrophosphate (10 γ per ml.) was included. Calcium was found to be necessary for the full activity of the enzyme. The mixture is incubated in test-tubes at 37°.

Air was used in the gas phase, since the amount of cysteine present was found to be sufficient to counteract the inhibitory effect of oxygen. Samples were removed at intervals and analyzed directly by the chemical method (3) and by bioassay after deproteinization (1).

The activity of the reaction mixture as determined by the bioassay rose from about 0.01 μeq. to 1.5 to 2.7 μeq. (270 to 470 γ) of acetylcholine per ml. at the end of 3 hours. The yield referred to 1 gm. of protein was equivalent to about 180 mg. of acetylcholine chloride per hour. At the concentrations finally used for bioassay (dilution factor of original solution 500 to 1000) the components of the reaction mixture added in-
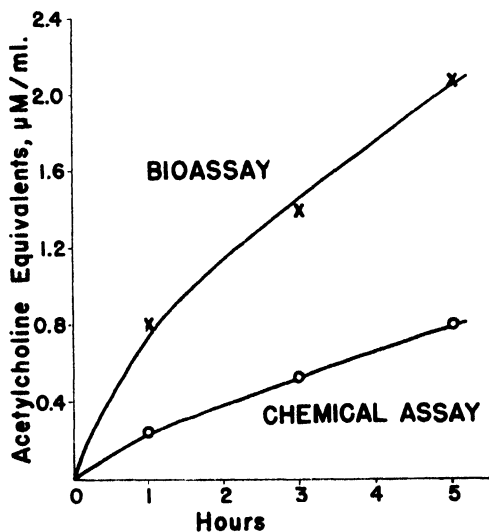
Fig. 1. Acetylcholine equivalents formed by enzymatic synthesis and determined by bioassay (frog rectus abdominis) and colorimetrically.

itially, separately or together, failed to elicit contraction of the frog rectus muscle and did not modify the response of the muscle to standard acetylcholine solutions.

The amount of acetylcholine was found to fall in a range which may be determined chemically by the colorimetric method recently introduced by Hestrin (3). This method is based on the reaction of O-acyl groups with hydroxylamine in alkaline medium and is thus suitable for the determination of the acetyl group of acetylcholine in the presence of choline and acetate. Less than half of the total biological activity of the enzymatically formed product could be accounted for by the chemical method (Fig. 1). Acetylcholine added to the reaction mixture at the outset or at the termination of incubation was recovered analytically without loss,

both with bioassay and with the chemical procedure. It thus follows that the greater part of the effect obtained in the bioassay is due to a substance which appears to have the same biological action as acetylcholine and which may be distinguished from the latter chemically.[2] In the absence of added choline, the product with biological activity was synthesized in amounts ranging between 0.4 and 0.6 μeq. of acetylcholine per ml. The chemical determination showed that no acetylcholine was formed when choline was omitted from the reaction mixture.

By further fractionation with ammonium sulfate, the quotient (microequivalents of biological activity)/(micromoles of acetylcholine) could be changed. A protein fraction precipitated at between 16 and 25 per cent ammonium sulfate synthesized acetylcholine preferentially and formed relatively little of the other biologically active substance (quotient, $\sim$1.3). When this protein fraction was incubated in a test system to which choline was not added, little or no biological activity was found. The protein fraction precipitated at between 25 and 36 per cent ammonium sulfate formed both active compounds (quotient, $\sim$1.5 to 3). This fraction also mediated a considerable synthesis of product with acetylcholine-like activity in a test system to which choline was not added.

Experiments to isolate and identify the new compound are in progress.

SUMMARY

1. The choline-acetylating system derived from rabbit brain was purified and concentrated by fractionation with ammonium sulfate. On incubation with choline, acetate, adenosine triphosphate, cysteine, coenzyme, and the ions potassium, calcium, and magnesium, this enzyme formed up to 2 to 3 μeq. of acetylcholine activity per ml.

2. The reaction product was tested by bioassay and with a chemical method for ester determination. A considerable discrepancy was observed between the two methods of analysis.

3. Evidence is presented that the acetylating enzyme system derived from brain forms, in addition to acetylcholine, a second product which exhibits the same biological activity as acetylcholine but differs from this ester chemically.

BIBLIOGRAPHY

1. Nachmansohn, D., and Machado, A. L., *J. Neurophysiol.*, **6**, 397 (1943).
2. Nachmansohn, D., and Weiss, M. S., *J. Biol. Chem.*, **172**, 677 (1948).
3. Hestrin, S., *J. Biol. Chem.*, **180**, 249 (1949).

[2] The biological properties of the enzymatically formed product are being investigated by Dr. S. Middleton and Dr. H. H. Middleton and will be reported separately.

ACYLATION REACTIONS MEDIATED BY PURIFIED ACETYL-CHOLINE ESTERASE*

By SHLOMO HESTRIN†

(From the Department of Neurology, College of Physicians and Surgeons, Columbia University, New York)

Earlier investigators have observed increases of pharmacological activity when crude tissue preparations are incubated in a concentrated solution of choline chloride and sodium acetate (1, 2). Inhibitors of esterase were shown to inhibit this reaction (3). It was suggested that a synthesis of acetylcholine had been effected in the test mixture to which inhibitor had not been added and that this synthesis had been mediated by an esterase. Findings presented below afford an experimental proof of this view.

An equilibrium

$$\frac{[\text{Acetylcholine}^+][\text{water}]}{[\text{Acetic acid}][\text{choline}^+]} \quad K$$
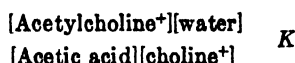
was demonstrated with the use of a highly purified preparation of acetylcholine esterase (4) as the catalyst. An equilibrium of a corresponding form was also observed in the presence of propionic acid. Alkaline hydroxylamine reagent (5) was used to estimate acetylcholine in the presence of an excess of the other participants of equilibrium. A representative experiment shown in Table I illustrates the attainment of equilibrium from the two reaction directions. The Nernst equilibrium constant (K), which is calculated in molarity, was found to have the value 0.25 at 23°. The free energy change of acetylcholine hydrolysis, estimated from the equation (6), $\Delta F^0 = -RT \ln (55.5/K)$, was found to be approximately -3100 calories.[1]

Formation of hydroxamic acid was observed when sodium acetate or propionate was incubated with hydroxylamine in the presence of acetylcholine esterase (see Table II). The acylation could be slowed or entirely prevented by inclusion of acetylcholine esterase inhibitors in the reaction mixture; *e.g.*, choline, prostigmine, or tetraethylpyrophosphate.

The findings provide experimental support for the suggestion by Lipmann (7) that the acylation of hydroxylamine by liver homogenate in the absence of added adenosine triphosphate is mediated by the activity of an O-acylhydrolase (lipase, esterase). It may be concluded that the acetyl-

TABLE I

Synthesis and Hydrolysis of Acetylcholine by Acetylcholine Esterase

The reaction mixture contained initially per ml. 1.4 mM of choline chloride, 1.4 mM of sodium acetate, and 41 mM of water. To Sample A no acetylcholine was added. To Sample B 8 μM of acetylcholine chloride per ml. were added at the outset. An amount of enzyme is present which would hydrolyze 2 gm. of acetylcholine chloride per hour in optimum conditions. The pH was set at 5.1 with hydrochloric acid; temperature 23°.

Sample	Acetylcholine, μM per ml., at different times			
	0 min.	60 min.	120 min.	180 min.
A	0	2.9	3.4	3.5
B	8.0	4.5	3.9	3.8

TABLE II

Synthesis of Acethydroxamic Acid by Acetylcholine Esterase

The reaction mixture contained per ml. 0.75 mM of sodium acetate and 1.00 mM of hydroxylamine. Sample A contained per ml. sufficient enzyme to hydrolyze 3 gm. of acetylcholine chloride per hour in optimum conditions. Sample B contained no enzyme. The pH was set with sodium hydroxide at 7.1; temperature 37°. At intervals aliquots of 0.5 ml. were brought to pH 1 with 1.0 N hydrochloric acid and diluted with 0.1 N hydrochloric acid. Acethydroxamic acid was determined colorimetrically by adding 1 ml. of 5 per cent ferric chloride in 0.1 N hydrochloric acid to 4 ml. of the diluted aliquot. The colored complex showed maximum absorption of light in the expected region, 500 to 520 mμ. A standard solution of acethydroxamic acid was prepared by allowing acetylcholine to react with an alkaline solution of hydroxylamine.

Sample	Acethydroxamic acid, μM per ml., at different times				
	0 min.	5 min.	55 min.	70 min.	83 min.
A	0	0.9	9.0	11.9	14.5
B	0		0		0

choline esterase mediates both N and O acylations. Substrate specificity and pH functions of these reactions will be described separately (8.)

SUMMARY

1. Purified acetylcholine esterase obtained from electrical tissue of *Electrophorus electricus* mediated a reversible hydrolysis of acetyl- and propionylcholine. The free energy change of hydrolysis of acetylcholine as estimated from the equilibrium constant was about -3100 calories.

2. Hydroxamic acid formation is observed when this enzyme is incubated with hydroxylamine in the presence of acetate or propionate. The reaction was inhibited by choline, prostigmine, and tetraethyl pyrophosphate. The esterase therefore is able to mediate both N and O acylation.

BIBLIOGRAPHY

1. Abderhalden, E., and Paffrath, II., *Fermentforschung*, **8,** 299 (1925).
2. Ammon, R., and Kwiatkowski, H., *Arch. ges. Physiol.*, **234,** 269 (1934).
3. Kwiatkowski, II., *Fermentforschung*, **15,** 138 (1936).
4. Rothenberg, M. A., and Nachmansohn, D., *J. Biol. Chem.*, **168,** 223 (1947).
5. Hestrin, S., *J. Biol. Chem.*, **180,** 249 (1949).
6. Meyerhof, O., and Green, H., *J. Biol. Chem.*, **178,** 655 (1949).
7. Lipmann, F., *Advances in Enzymol.*, **6,** 257, (1946).
8. Hestrin, S., *Biochem. et biophys. acta*, in press.

STARCHES AND THEIR DERIVATIVES AS ADSORBENTS FOR MALT α-AMYLASE*

BY SIGMUND SCHWIMMER AND A. K. BALLS

(From the Enzyme Research Division, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Albany, California)

Some 40 years ago Starkenstein (1) demonstrated that liver amylase is removed from solution by a suspension of insoluble starch. Similar adsorption was later observed by Ambard (2) with salivary amylase and by Chodat and Philia (3) with malt "diastase." Boekestein (4), using pancreatic α-amylase, reported a detailed study on the effects of temperature, pH, and adsorbent concentration on its adsorption by rice, wheat, and potato starch. No experimental data are given. Holmberg (5, 6) showed that malt α-amylase could be separated from the β-amylase by adsorption of the former on starch from cold 50 per cent ethanol in the presence of maltose. His results showed that the adsorption increased with increasing alcohol concentration, and at lowered pH and temperature. Blom, Bak, and Brae (7) employed adsorption on starch granules as one means of detecting the presence of more than one amylase in a given source. Thus the relationships between reducing value, viscosity, and dextrinizing changes were not changed by partial adsorption of the amylases from pancreas, bacteria, and molds, whereas these relationships changed when malt amylase was used. Tokuoka (8) was enabled to purify mold α-amylase by adsorption on steamed rice in the presence of alcohol during a Koji fermentation. The amylase of *Clostridium butylicum* was purified by Hockenhull and Herbert (9) by adsorption on potato starch in the presence of alcohol. More recently Schwimmer and Balls (10) found that adsorption of partially purified malt α-amylase on a column of wheat starch at 5° in the presence of 40 per cent ethanol was an essential step in the isolation and crystallization of the enzyme. This adsorption was found to be a highly selective process, evidently almost completely specific for the α-enzyme protein present in the partially purified preparations. The enzyme could be eluted from the starch by means of water (containing calcium) at room temperature.

Our use of wheat starch for the adsorption of malt α-amylase was, however, an accidental choice; it was the first starch tried. Therefore the probability was good that other starches would prove significantly better

---

or worse for this adsorption, and that fractions of starch, such as amylose and amylopectin, would also differ in efficiency. The following experiments showed that starches do vary and led us to a number of general conclusions that are summarized later.

Materials and Methods

Malt α-amylase was determined as described by Schwimmer (11). A stock solution of partially purified enzyme was made from a concentrated malt extract.[1] The extract was heated to 70° for 15 minutes and filtered; 250 gm. per liter of ammonium sulfate were added to the filtrate and the precipitate obtained was then dissolved in 200 ml. of saturated calcium sulfate solution. After dialysis at 5° for several days against successive changes of saturated calcium sulfate water and finally against 0.5 per cent calcium chloride solution, the enzyme preparation was filtered and used for adsorption studies. Such stock enzyme solutions contained 200 to 240 units of α-amylase per ml. and had a specific activity of 84 units per mg. of protein nitrogen (compared with 390 for the crystalline enzyme). 0.005 ml. of the stock enzyme, when added to 15 ml. of 1.33 per cent soluble starch at 30°, pH 4.75, would hydrolyze 6.64 per cent of the total glycosidic bonds in 10 minutes.

The starch granules, modified starches, and starch fractions used as adsorbents will be described under the appropriate sections.[2]

Adsorption was carried out at 0° in the presence of 40 per cent alcohol and 0.5 per cent calcium chloride. To 3 ml. of the stock enzyme solution, 7 ml. of 0.5 per cent calcium chloride were added and then 10 ml. of 80 per cent ethanol containing 0.5 per cent calcium chloride. An appropriate amount of solid adsorbent (determined by a preliminary run) was added to the alcoholic solution and stirred with a magnetic stirrer. For analysis 2 ml. aliquots of the suspension were pipetted into a cold centrifuge tube and centrifuged at 0° for 3 minutes at 3200 revolutions per minute, corresponding to a mean centrifugal field of about 1100 times gravity. The time of adsorption was taken as the interval between the addition of adsorbent and the beginning of centrifuging. After centrifuging, the supernatant liquid was assayed for α-amylase and compared with a valid control containing no starch. No substantial change was observed in such controls during the time required for the operations. The percentage of enzyme adsorbed was calculated from assays of the supernatant liquid.

The results are expressed in terms of the amount of adsorbent which would give 50 per cent adsorption in 2 minutes (unless otherwise stated) under the specified conditions. This short interval was selected because it was long enough for most of the adsorption to take place, yet short enough to minimize possible penetration or slight digestion of the adsorbent by the enzyme. The efficiency of the adsorbent is given as the weight of adsorbent required for 50 per cent adsorption, compared with the weight of raw wheat starch required to do the same thing. (Under the conditions outlined, 5.1 gm. of our raw wheat starch adsorbed half the enzyme; therefore, the efficiency of another adsorbent is expressed as 5.1 divided by the weight of the other adsorbent required to adsorb half the amylase.) As is shown

TABLE I

Effect of Purity on Adsorption of α-Amylase by Freshly Precipitated Starch Suspensions

Treatment	Specific activity $\left[\dfrac{\alpha\, u.}{PN}\right]$	Total soluble activity of adsorption systems*		Per cent enzyme adsorbed
		Before adsorption [α u.]	After adsorption [α u.]	
Malt extract heated, filtered, precipitated by saturation with ammonium sulfate, dialyzed	15	531	187	64.7
Stock enzyme	80	558	195	65.0
Dialyzed solution of crystalline α-amylase	390	607	215	64.6

* Weight of starch present = 120 mg.

in Table I, the degree of purity of the enzyme did not materially affect its adsorbability by the foregoing technique.

RESULTS AND DISCUSSION

Adsorption Efficiencies of Raw Starch Granules—The varieties of raw starch granules used in the adsorption experiments are listed in Table II according to average diameter obtained by microscopic observation similar to that of Wolf *et al.* (12), except that the sample under observation consisted of fewer granules. The listing of the adsorption efficiencies of these starches for α-amylase shows a definite correlation between diameter (and therefore specific surface area) and efficiency of adsorption.

Four exceptions to this statement appear in Table II: the starches from waxy maize, wrinkled pea, wheat, and the "amylodextrin" fraction of wheat flour. Although the average diameter of the granules of wheat starch is greater than that, for instance, of corn, it must be remembered that the wheat starch granule is disk-shaped and would have a greater

specific surface than a spheroid granule of the same diameter. Furthermore the high proportion in wheat starch of the amylodextrin fraction, 10 to 15 per cent (13), would again tend to increase the specific surface area. The higher efficiency of the amylodextrin fraction may be explained by the substantial amount of broken kernels therein. Therefore, the only real discrepancies are in the waxy maize, which contains no amylose (14), and wrinkled pea starch, which contains about 3 times as much amylose as is usually found in starches (15). At least part of this increase in adsorption efficiency may be due to the compound nature of the granule,

TABLE II

Adsorption Efficiencies of Starch Granules As Adsorbents for α-Amylase

Source of starch granules	Granule size		Adsorption efficiencies
	Range	Average	
Canna.. .	35–150	85	0.2
Potato. ...	15–100	65	0.3
Banana.....	20– 80	50	0.3
Smooth pea.	15– 40	30	0.6
Wheat.......	20– 35 ⎱ 2– 10*⎰	25 5	1.0
Wrinkled pea	15– 35	25	8.0
Corn	5– 25	15	0.8
Cassava	5– 35	15	0.8
Bean.......	10– 30	15	0.7
Waxy maize...	5– 25	15	2.5
Sweet potato	10– 20	15	0.7
Oats..	5– 15	10	0.9
Amylodextrin granules from wheat . .|	2– 10	5	2.1
Rice.....	3– 8	5	1.5
Dasheen.	1– 6	3	1.6

* Represents the size of the "amylodextrin" fraction, which was about 10 per cent of the total.

"composed of several simple wedge-shape granules which are the fragments formed by cleavage" (15).

Hence we may conclude that for starches of the usual amylose content, consisting of simple granules, the efficiency of adsorption depends on the surface area of the granule.

Adsorption Efficiency of Treated Starches—It is evident from Table III that adsorption efficiency can be increased by various treatments of the starch granule, apparently when this treatment results in increased surface area. Thus a sample finely ground in a ball mill showed a large extent of granule disintegration. On the other hand, relatively mild treat-

ment, which did not greatly change the granule structure (light ball milling, solvent extraction, acid treatment), also increased the efficiency of adsorption but to no marked extent. It is not surprising, therefore, if one assumes that the adsorption efficiency is primarily a function of surface, that the most efficient adsorbent turned out to be Lintner starch freshly

TABLE III

Adsorption Efficiency of Modified or Treated Starch Granules

Starch	Treatment	Adsorption efficiency
Wheat	None	1.0
"	Coarsely ground in ball mill*	4.4
"	Finely " " " " †	10.6
Corn	None	0.8
"	Defatted	1.1
Potato	None	0.3·
"	Acid (Lintner, soluble)	0.5
"	Precipitated from solution by alcohol	69.0

* 2 kilos ground for 7 days at 5 revolutions per minute.
† 1 kilo ground for 7 days at 13 revolutions per minute.

TABLE IV

Adsorption Efficiency of Various Starch Fractions

	Source	Adsorption efficiency
Amylose	Corn	40
"	Wheat	49
"	Potato	11
"	Wrinkled pea	27
Amylopectin	Corn	12
"	Wheat	16
"	Potato	2.8
β-Limit dextrin	Corn amylopectin	17
Glycogen	As solid	76
"	Freshly precipitated	376

precipitated by alcohol, to which enzyme was added to give the standard adsorption mixture.

Adsorption Efficiencies of Starch Fractions—In view of the importance of particle size as just demonstrated, the meaning of results with starch fractions must be somewhat elastic, because it was not possible to subdivide them all equally well. However, all such material was first shaken (not forced) through a No. 270 sieve (screen opening 0.02 mm. sq.).

As is seen in Table IV, the amyloses tried were better adsorbents than the amylopectins. However, the high efficiencies observed for the amylopectin fractions are, in a sense, surprising, inasmuch as the addition of these fractions to the alcoholic enzyme solution resulted in a dispersion consisting of relatively coarse rubber-like particles, indicating that the total surface area was much less than that of the amylose-ethanol suspensions. Although β-limit dextrin also gave similar coarse suspensions, it adsorbed better than did amylopectin, presumably owing to the lack of linear maltosidic chains at the periphery of the amylopectin moiety.

One might conclude from these observations that, *if* amylopectin could be dispersed as finely as amylose, it would adsorb much better, and the astonishing adsorptive capacity of freshly precipitated glycogen bears this conclusion out. Glycogen, whose molecule also consists of a highly ramified glucosido polymeric structure, and gives a fine granular-like suspension in 40 per cent alcohol, showed an extremely high efficiency, as shown in Table IV. Furthermore (Table II) the waxy maize granule is about 3 times as efficient as the corn-starch granule, which it resembles closely in gross structure and size. Inspection of the data in Table IV shows that wheat and corn amyloses are much better adsorbers than is potato amylose. The relative order of efficiency parallels rather closely the order of the rate and extent of degradation from dispersions of these fractions as described by Whistler and Johnson (16). They explain these differences as being due to the difference in the average chain length (as determined by viscosity), potato amylose having the greatest length. Furthermore, Schwimmer in a study of the kinetics of hydrolysis of these amyloses by crystalline malt α-amylase (to be reported in a subsequent paper) has demonstrated that the maximum velocity attainable by a given enzyme concentration and the affinity between enzyme and substrate follows the order: potato amylose > corn amylose > wheat amylose (*i.e.*, in the same order as the chain length of these fractions).

These results suggest that the α-amylase is adsorbed most efficiently on substrates which consist of smaller glycosidic chains. The lower limit of chain length for the amylose, according to this hypothesis, would be that at which the dextrins become soluble in 40 per cent alcohol. For branched chain polymers, this limit is much lower, since the small dextrins are, in effect, linked into the large polymer by α-1,6-glucosido linkages. Thus glycogen with an average chain length (between two successive branching points) of perhaps 8 to 12 glucosidic residues is a better adsorber than β-limit dextrin, with an average chain length of about 20 to 25 residues.

The idea that small chains are better adsorbents is in accord with the results obtained with freshly precipitated fractions of partly digested starch. Table V shows the adsorption obtained when soluble (Lintner)

starch was digested with a negligible quantity of enzymes and precipitated with alcohol. Enzyme solution in the amount required to bring the alcohol concentration to 40 per cent, as in the usual procedure, was then added after precipitation had taken place. As can be seen from Table V, the actual percentage of amylase adsorbed increased with increasing digestion until 2.5 per cent of the maximum possible hydrolysis was reached. Thereafter it decreased. However, the actual amount of adsorbent was decreasing continuously during the first part of the digestion, as a result of increasing solubility of the split-products in dilute alcohol. Thus the values given in Table V do not reflect the high efficiencies probable for

TABLE V

Partially Hydrolyzed Starch As Adsorbent for α-Amylase

Time of hydrolysis	Per cent glycosidic bonds hydrolyzed	Per cent adsorption
min.		
0	0.0	53.3
1	1.7	58.7
2	2.5	67.9
4	4.4	53.3
6	5.9	51.0

The reaction mixture consisted of 2 units of enzyme acting on 400 mg. of soluble starch in a volume of 30 ml. of starch at 30°, pH 4.7. These are the usual conditions of assay, and the enzyme present is negligible compared with that added later and present in the adsorption experiment. At the indicated times, 10 ml. of 80 per cent ethanol were added to 6 ml. of the digestion mixture (equivalent to 80 mg. of starch). The suspension was cooled and 4 ml. of enzyme (592 units) to be adsorbed were added, making the total volume 20 ml. and the alcohol concentration 40 per cent, as in the standard adsorption procedure. The per cent of glycosidic bonds hydrolyzed was estimated from the color value of the digest after the addition of iodine, as previously described (10).

the partially digested starch. This is very striking at 5.9 per cent digestion, at which only a very light turbidity was obtained in 40 per cent ethanol; yet the degree of enzyme adsorption was only slightly lower than that of the untreated control. As will appear later, some part of this increased efficiency may be due to the effect of the soluble dextrins formed, but their effect was pronounced only in greater concentrations than could occur here.

The assumption that, per unit surface, those adsorbents with shorter chain lengths are more efficient implies the rather reasonable hypothesis that only a limited number of enzyme molecules can combine with one maltosidic chain, apparently regardless of its length.

Effect of Time and Fineness on Adsorption—When the starch used as

adsorbent is finely enough divided, most of the adsorption takes place within 2 minutes. It then rather slowly approaches a maximum within 30 minutes, but if carried out long enough, the extent of adsorption decreases (Fig. 1). If, however, coarse particles are used, the adsorption
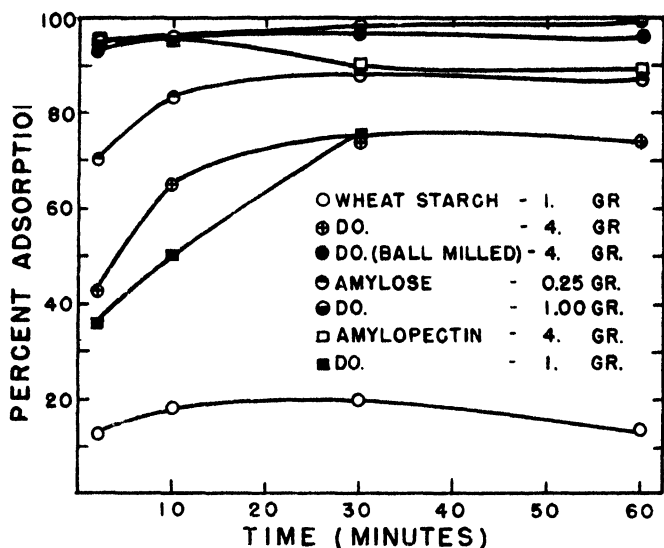
FIG. 1. Adsorption of malt α-amylase on various starches as a function of time

TABLE VI

Effect of Particle Size on Rate of Adsorption

Starch derivative	Weight	Per cent adsorption in		
		2 min.	30 min.	60 min.
	gm			
Amylose, through sieve No. 270	0.25	71	88	87
" " " " 60	0.25	11	67	74
" " " " 20	0.25	11	23	35
Amylopectin, through sieve No. 270	1.00	73	61	
" not through sieve No. 140	1.00	35	77	

rises slowly (Table VI). In Table VI, the adsorption of two different samples of amylose which were recrystallized, dried, and passed through relatively coarse sieves showed a slow but increasing adsorption with time. One recrystallized amylose sample consisted of particles which passed through a No. 20 sieve, another sample passed through a No. 60 sieve, and a third sample through a No. 270 sieve. The "coarse" amylopectin
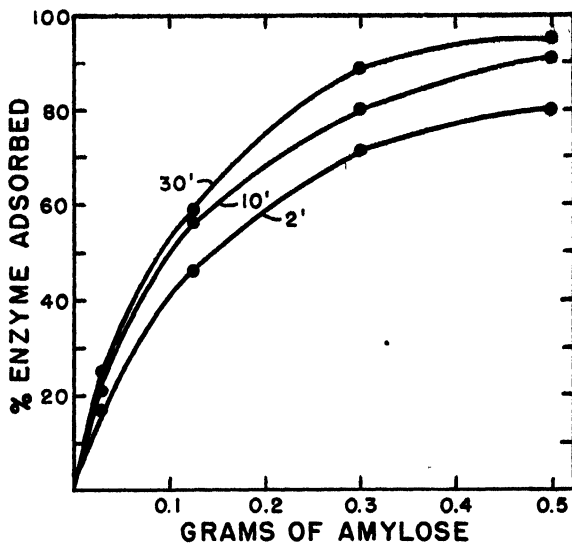
Fɪɢ. 2. Adsorption of malt α-amylase by corn amylose as a function of adsorbent concentration at low amylose concentrations for various times.
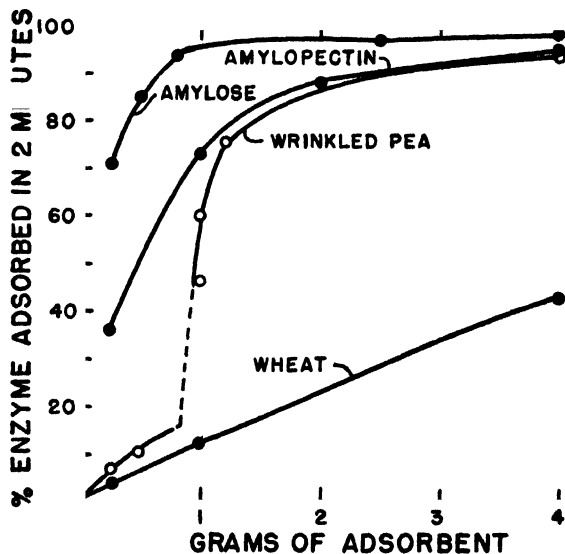
Fɪɢ. 3. Adsorption (2 minutes) of malt α-amylase on large amounts of various starches as a function of adsorbent concentration.

was obtained from the original sample by collecting those particles which would not pass through a No. 140 sieve. In this case, although the rate of adsorption was much slower for the larger particles, the ultimate amount

of enzyme adsorbed was actually greater than the maximum amount adsorbed on the fine particles. This suggests that, given time enough, some penetration of the adsorbent by the enzyme occurs.

Effect of Adsorbent Concentration—The effect of low concentrations of amylose on the amount of enzyme adsorbed at various times is shown in Fig. 2. Comparison of this adsorption with that of amylopectin, wheat starch granules, and wrinkled pea starch granules at higher concentrations is shown in Fig. 3. It can be seen from these data that both amylose and
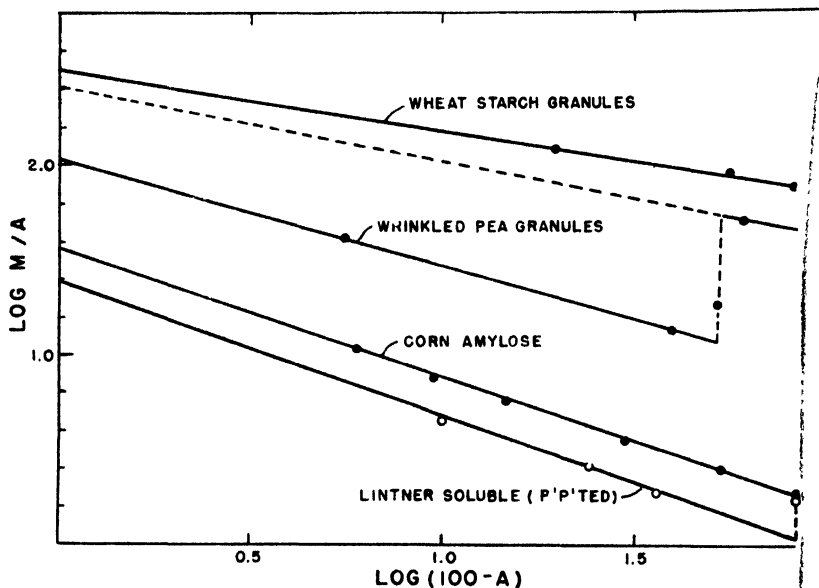
FIG. 4. Adsorption isotherm for various starches. The Freundlich equat[ion] used in the form $M/A = K(100 - A)^N$; where M = the weight of adsorbent in[ ] A is the percentage of enzyme adsorbed, and $(100 - A)$ is the amount rem[a] in the supernatant liquid. Hence $\log M/A = N \log (100 - A) + \log K$.

amylopectin are consistently better adsorbents than wheat starch ov[er a] wide range of concentration, while wrinkled pea starch is a good adso[rbent] only at high concentrations. The strange behavior of wrinkled pea s[tarch] is illustrated in another way by comparing adsorption isotherms for [pea] starch with that of wheat starch granules and that of corn amylose [(Fig.] 4). The slopes of the curves for these isotherms are all about the s[ame] but the curve for pea starch exhibits a break at a concentration of abo[ut] gm. As is to be expected, the intercepts on the ordinate of the ex[tra]polated curves show lower values for the better adsorbents.

Effect of Digestion Products on Adsorption by Freshly Precipitated Starc[h]

Table VII shows that the end-products of starch digestion exert a rather significant influence on the adsorption. Whereas, maltose and especially limit dextrin inhibit, α-dextrin actually enhances the adsorption considerably. These results can be interpreted in the light of the inhibitory effect of these substances on the hydrolysis of soluble starch by crystalline α-amylase. Such considerations led to a study of the kinetics of the inhibition, wherein it has been found that, while α-dextrin inhibits amylolysis by malt α-amylase competitively, maltose inhibits non-competitively. These and similar results will be reported in a subsequent paper.

Since maltose inhibits α-amylase activity. by a non-competitive mecha- 'n and since maltose, but not α-dextrin, inhibits amylase adsorption, we

TABLE VII

Influence of Starch Digestion Products on Adsorption

.ction product per 20 ml.	Per cent adsorption		
	Maltose	Limit dextrin	α-Dextrin
Fı *mg.* conce 0	25.1	25.1	25.1
10	22.3	11.4	25.5 .
50	9.0	4.0	26.6
250	8.1	0.9	37.6

e adsorption system contained 545 α-amylase units and 40 mg. of freshly pre- .ted starch in a total of 20 ml. of 40 per cent ethanol. Limit dextrin is the ermentable material obtained from the exhaustive hydrolysis of starch by extract, as described by Kneen and Spoerl (17). α-Dextrin was obtained an α-amylase starch digest by stopping the reaction with HCl at 10 per cent ng of total glycosidic bonds (just enough to give very little color with iodine). it was neutralized and precipitated with 4 volumes of ethanol, the product -ied *in vacuo* and used as α-limit dextrin.

:onclude that the site for adsorption of the enzyme on the starch is ie same as the site of union of enzyme with substrate to form a hydro-le complex. The enhancement of adsorption by α-dextrin can then terpreted as follows: The enzyme, after combining at one "active r" with the dextrin, is still free to be adsorbed on the starch through a ł "active center." The adsorbed dextrin will then present a new :e for further adsorption. This adsorption will then be more efficient ' first assumption is correct; *i.e.*, that dextrins with shorter chain ıs are better adsorbents.

SUMMARY

The adsorption of malt α-amylase in 40 per cent ethanol at 0° on les, modifications, and fractions of starch has been studied.

2. The efficiency of adsorption is inversely proportional to the granule size, or, more precisely, to the surface area of the granules.

3. Various kinds of treatment of the granule increase this efficiency somewhat. The most effective adsorbents are starches freshly precipitated from solution with alcohol.

4. The adsorption obeys, within limits, the Freundlich adsorption isotherm.

5. Starch fractions (*i.e.*, amylopectin, dextrins, amylose, glycogen) exhibit a different higher order of magnitude of adsorption than do the granules. The exact degree of efficiency depends upon the structure, the source, and fineness of subdivision of the substance used.

6. The degree of adsorption is independent of the state of purity of the enzyme.

7. Maltose and limit dextrin decrease, whereas α-dextrin increases the extent of adsorption.

8. It is concluded from the data obtained that the determining factor in this adsorption is the total number of insoluble maltosidic chains available to the enzyme in solution. This number in turn depends directly upon the surface area of the adsorbent and inversely upon the solubility and chain length, but not necessarily on molecular size.

BIBLIOGRAPHY

1. Starkenstein, E., *Biochem. Z.*, **24**, 210 (1910).
2. Ambard, L., *Bull. Soc. chim. biol.*, **3**, 51 (1921).
3. Chodat, F., and Philia, M., *Compt. rend. Soc. phys. et hist. nat Genève*, **41, 118** (1924)
4. Boekestein, P. T., *Acta Brev. Neerl.*, **2**, 132 (1932).
5. Holmberg, O., *Ark. Kemi, Mineral. o. Geol.*, **11 B**, No. 4 (1933).
6. Holmberg, O., *Biochem. Z.*, **258**, 134 (1933).
7. Blom, J., Bak, A., and Brae, B., *Z. physiol. Chem.*, **250**, 104 (1937).
8. Tokuoka, Y., *J. Agr. Chem. Soc. Japan*, **13**, 586 (1937).
9. Hockenhull, J. D. J., and Herbert, D., *Biochem. J.*, **39**, 102 (1945).
10. Schwimmer, S., and Balls, A. K., *J. Biol. Chem.*, **179**, 1063 (1949).
11. Schwimmer, S., *Cereal Chem.*, **24**, 315 (1947).
12. Wolf, M. J., MacMasters, M. M., Hubbard, J. E., and Rist, C. E., *Cereal Chem.*, **25**, 312 (1948).
13. MacMasters, M. M., and Hilbert, G. E., *Cereal Chem.*, **21**, 548 (1944).
14. Cook, L. M., and Kerr, R. W., in Kerr, R. W., Chemistry and industry of starch, New York (1944).
15. Hilbert, G. E., and MacMasters, M. M., *J. Biol. Chem.*, **162**, 229 (1946).
16. Whistler, R. L., and Johnson, C., *Cereal Chem.*, **25**, 418 (1948).
17. Kneen, E., and Spoerl, J. M., *Proc. Am. Soc. Brew. Chem.* (1948).

THE EFFECT OF 2,4-DINITROPHENOL ON HEXOKINASE AND GLYCOGENOLYSIS*

BY F. T. PIERCE† AND JOHN FIELD

(From the Department of Physiology, Stanford University School of Medicine, Stanford University, California)

The augmentation of cell respiration caused by low concentrations of 2,4-dinitrophenol (DNP) and related substituted phenols is well known (cf. Peiss and Field (1) for review). At higher concentrations, this substance acts as an inhibitor, and it has been suggested that the augmentation of respiration at low concentrations is an anomalous aspect of inhibition. Peiss and Field (1) have shown that the absolute rate of respiration of rat brain homogenates operating at possible maximum activity is approximately equal to the respiration of tissue slices with the maximum stimulatory concentration of DNP. Since homogenates are thought to operate free of normal cell brakes or inhibitors (the brake being removed by dilution or destroyed when the integrity of the cell is disrupted (2)), it has been postulated that DNP operates by inhibiting this natural brake. This would permit the tissue slice to oxidize glucose at its maximum capacity when in contact with DNP.

It has been shown that insulin increases glucose utilization and glycogen formation in isolated diaphragm (3–8). The enzyme hexokinase which catalyzes the reaction, glucose + adenosine triphosphate $\rightarrow$ glucose-6-phosphate + adenosine diphosphate, must therefore be influenced by this hormone, either directly or indirectly (3, 9–17). Since the hexokinase reaction is irreversible, measurements of glucose uptake should be a measure of the activity of this enzyme (13). If DNP were found to accelerate the hexokinase reaction (insulin-like effect), this would contribute to the clarification of its position as an accelerator of tissue respiration.

Consequently measurements of glucose uptake and glycogen balance in rat diaphragm in the presence and absence of maximum stimulatory concentrations of DNP were undertaken. The results are reported in this paper.

Methods

The diaphragms from fifteen adult albino rats of the Slonaker-Wistar strain were used in this study. The stock diet of these rats was Purina

fox checkers given *ad libitum*, and they were not fasted prior to use. Both sexes were used in about equal numbers, and no significant difference was noted between them. The average body weight was 200 gm. No animals weighing over 240 gm. were used, since the diaphragms were so thick as to impair oxygen diffusion. Smaller rats were not used because the diaphragms did not supply enough material for carbohydrate balance measurements.

The rats were decapitated, and each hemidiaphragm removed, as described by Krahl and Cori (13). The pieces of diaphragm were transferred to a Petri dish set in cracked ice. This was kept humid by a piece of filter paper moistened with Ringer's solution stuck on the under surface of the lid. Pieces of muscle were then weighed on the micro torsion balance and put at once into chilled respirometer vessels which contained the necessary solutions. By this method, the tissue was kept in a cold and moist environment until oxygenated, and anaerobic metabolism was minimized (18). The average weight of tissue was 60 mg., and 60 mg. or more of each diaphragm were used to determine the initial glycogen content.

The suspension medium was Krebs' Ringer-phosphate (19–22) and glucose with or without DNP to make a total volume of 1 ml. The glucose concentration used was 100 mg. per cent. Although glucose utilization is higher at higher concentrations (300 to 500 mg. per cent) (5), the lower level was chosen as that corresponding more nearly to the normal blood sugar level. The pH of the medium was 7.15 to 7.20 in all experiments.

DNP was added to Krebs' Ringer-phosphate and glucose to give a final concentration of 4.76×10^{-5} M, and 1 ml. of this solution was put into alternate vessels. In this manner approximately equal numbers of pieces of diaphragm of each rat were incubated with and without DNP. Previous experiments had shown that this concentration of DNP produced maximum augmentation of oxygen uptake in diaphragm.

The center wells of the vessels were filled with 0.1 ml. of 5 per cent KOH to absorb the respiratory CO_2, and the gas phase was 100 per cent oxygen. The respirometer vessels were loaded with tissue and placed on the Warburg manometers. They were then oxygenated at room temperature for 3 minutes and placed in a constant temperature bath at $37.5 \pm 0.01°$. Here they were shaken for 2 hours at 106 cycles per minute.

At the end of this time, the pieces of diaphragm were rapidly removed from the flasks and placed in 30 per cent KOH for determination of glycogen by the method of Good, Kramer, and Somogyi (23), as modified by subsequent workers (24, 25). 0.2 ml. of the residual medium was pipetted into zinc sulfate for protein precipitation by the method of Somogyi (26). All glucose determinations were made in duplicate.

violet absorption spectra of strong sulfuric acid solutions of the saccharic' for analytical purposes and we have now demonstrated that this procedure is a practical one in a number of cases. The desirability of using 79 per cent sulfuric acid as a medium was suggested by earlier studies in these laboratories (6), and it will be noted that sulfuric acid of this concentration is still an excellent solvent for practically all polyhydroxy compounds (9).

Wave Length in Millimicrons
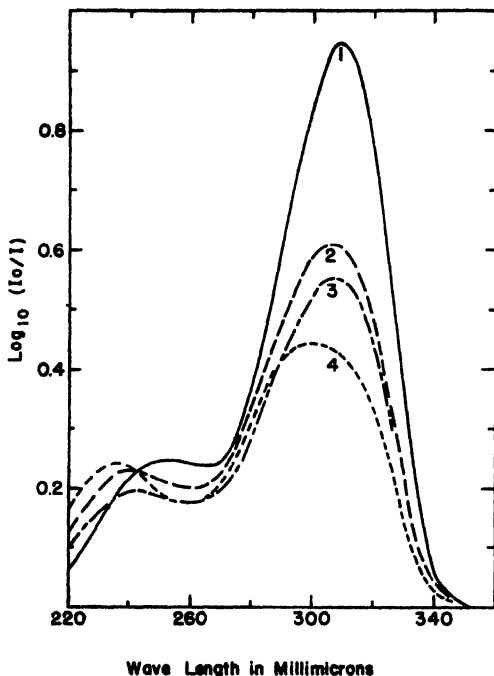
FIG. 1. Spectra of heated solutions of the aldopentoses in 79 per cent sulfuric acid. Curve 1, 100 γ of xylose; Curve 2, 100 γ of ribose; Curve 3, 100 γ of arabinose; Curve 4, 100 γ of lyxose.

EXPERIMENTAL

84 per cent (by weight) sulfuric acid was prepared from reagent grade acid. The carbohydrate solutions contained 100 γ of anhydrous compound per ml., account being taken of water of crystallization whenever necessary. The glucosamine hydrochloride solution contained 100 γ of free base per ml. The identity and purity of the individual compounds were checked by a determination of their optical rotation and their melting points.

9 ml. portions of 84 per cent sulfuric acid contained in 15 $\times$ 150 mm.

A SPECTROPHOTOMETRIC STUDY OF THE BEHAVIOR OF CARBOHYDRATES IN SEVENTY-NINE PER CENT SULFURIC ACID*

BY MIYOSHI IKAWA AND CARL NIEMANN

(From the Gates and Crellin Laboratories of Chemistry,† California Institute of Technology, Pasadena)

Carbohydrates *per se* do not exhibit significant specific absorption in the 210 to 400 mμ region (1, 2), but, when exposed to the action of strong mineral acids, they are transformed into products which do absorb specifically in this region and which will react with a variety of reagents to give colored addition products. This latter fact has been utilized in the development of colorimetric methods suitable for the identification and estimation of mono-, di-, and polysaccharides, as, for example, in the carbazole-sulfuric acid method described by Dische (3, 4). Although a considerable amount of information is available with respect to the spectral characteristics of the various colored addition products (5–7), relatively little is known about the absorption spectra of solutions of carbohydrates in strong sulfuric acid, the medium used in most of these procedures.

Bandow (8) determined the absorption spectra of solutions of a number of sugars in concentrated sulfuric acid after the solutions had been allowed to remain at room temperature for a day and noted that, while the spectra were not superimposable, the absorption curves for the hexoses, pentoses, and glucuronic acid were similar to that of furfural determined under the same conditions. In the course of a systematic study (6) of the variables influencing the Dische carbazole method the spectra of solutions of glucose, galactose, mannose, fructose, and *N*-acetylglucosamine in 79 per cent sulfuric acid were determined, and it was noted (6) that with fructose the strong extinction, observed with solutions that had stood at 25° for 40 minutes, decreased when these solutions were subsequently heated at 100° for 15 minutes. In contrast to the above, solutions of the aldohexoses exhibited no significant specific absorption when allowed to stand at 25° for 40 minutes but did absorb strongly in the 315 mμ region after the solutions had been heated at 100° for 15 minutes.

The above observations suggested the possibility of utilizing the ultra-

---

be due to an interference in the enzyme systems necessary for bone development indirectly as a result of a lowered liver copper, and also by a possible direct poisoning effect, as has already been observed with certain phosphatases (13, 14).

SUMMARY

The dependence of low level molybdenum toxicity upon the dietary copper intake of the rat has been demonstrated.

The tissue distribution of Mo^{99} intravenously injected and orally administered to the bovine is presented, and the similarity between the behavior of phosphorus and molybdenum is shown.

The interrelationships of molybdenum, copper, and phosphorus in the rat have been investigated with the radioisotopes of these elements. The results suggest that the toxic action of molybdenum may be due to the following mechanisms, which are listed in probable order of decreasing importance: (a) Interference, due to a lowered liver copper, in enzyme systems necessary for skeletal metabolism; (b) inhibition of these enzyme systems by molybdenum; and (c) competition between phosphorus and molybdenum for deposition in the bone. It seems unlikely that the toxic action of molybdenum can be accounted for simply by a complex formation which renders other elements unavailable.

BIBLIOGRAPHY

1. Ferguson, W. S., Lewis, A. H., and Watson, S. J., *Nature*, **141,** 553 (1938).
2. Russell, F. C., *Imp. Bur. Animal Nutr., Tech. Communicat., No. 15,* May (1944).
3. Britton, J. W., and Goss, H. J., *J. Am. Vet. Med. Assn.*, **108,** 176 (1946).
4. Barshad, I., *Soil Sc.*, **66,** 187 (1948).
5. Davis, G. K., and Hannan, H., *J. Animal Sc..* **6,** 484 (1947).
6. Fairhall, L. T., Dunn, R. C., Sharpless, N. E., and Pritchard, E. A., *Pub. Health Bull., U. S. P. H. S., No. 293* (1945).
7. Neilands, J. B., Strong, F. M., and Elvehjem, C. A., *J. Biol. Chem.*, **172,** 431 (1948).
8. Comar, C. L., and Davis, G. K., *Arch. Biochem.*, **12,** 257 (1947).
9. Comar, C. L., *Nucleonics*, **3,** No. 3, 32 (1948).
10. Comar, C. L., Davis, G. K., and Singer, L., *J. Biol. Chem.*, **174,** 905 (1948).
11. Dick, A. T., and Bull, L. B., *Australian Vet. J.*, **21,** 70 (1945).
12. McGowan, J. C., and Brian, P. W., *Nature*, **159,** 373 (1947).
13. Courtois, J., and Bossard, M., *Bull. Soc. chim. biol.*, **26,** 464 (1944).
14. Bossard, M., *Bull. Soc. chim. biol.*, **29,** 218 (1947).

on pastures in Florida having an unfavorable molybdenum-copper ratio have shown values of around 10 p.p.m. of copper and up to 10 p.p.m. of molybdenum on the dry basis; normal liver values have been found to range from 80 to 130 p.p.m. of copper, and 0 to 2 p.p.m. of molybdenum. This is in agreement with the report of Dick and Bull (11) who found similar values for the livers of cows on a high molybdenum intake over a period of 3 years. They also showed that, when molybdenum intake of sheep was increased to 10 or 100 mg. per day, the copper concentration in the liver was significantly reduced.

<div style="text-align:center">DISCUSSION</div>

A theory for the copper-molybdenum action, which has recently been suggested by McGowan and Brian (12), is as follows: The activity of bacteria in the gastrointestinal tract is controlled by catechols; molybdates reduce the effective concentration of catechols by formation of complexes and the uncontrolled bacterial activity becomes excessive and causes the condition which is manifested by extreme diarrhea; the therapeutic effect of copper is due to its toxic action on the bacteria. In preliminary investigations, cows exhibiting severe diarrhea associated with an unfavorable copper-molybdenum ratio were given large quantities of sulfathiazole (up to 3 grains per pound) by Davis and Kidder.[2] No effects on the diarrhea or other symptoms were observed. This would indicate that the symptoms are probably not caused simply by uncontrolled bacterial activity.

The interference in bone metabolism most marked with molybdenum intake cannot be accounted for by the formation of an unavailable molybdenum-phosphorus complex. The data indicate that simultaneous administration of molybdenum to the rat did not decrease phosphorus utilization. Either molybdenum or phosphorus administered simultaneously with copper decreased the liver accumulation of copper. Since relatively high phosphorus diets, otherwise normal, do not cause bone changes nor is the condition so extreme in uncomplicated copper deficiency, it is suggested that molybdenum has some toxic action over and above the reduction of liver copper.

In attempting to clarify the action of molybdenum in the animal, the following experimental facts should be taken into consideration: (a) Molybdenum is accumulated in the bone at about the same rate as phosphorus, (b) molybdenum tends to reduce the copper storage in the liver, (c) molybdenum toxicity is apparently overcome by administration of excess copper, and (d) alkaline blood phosphatase values of cattle are raised when molybdenum-copper imbalance exists. Perhaps the action of molybdenum may

[2] Davis, G. K., and Kidder, R. W., unpublished data.

there is a tendency for the phosphorus accumulation to be decreased. This was confirmed by another experiment with two groups of four mature rats which had been on high and low molybdenum diets for some time. The rats on the high molybdenum diet showed relative P^{32} concentration values for the blood, liver, kidney, and bone of 0.1, 1.1, 0.7, and 2.0, respectively; the corresponding values for the rats on a low molybdenum ration were 0.2, 1.7, 1.2, and 3.5.

Effect of Molybdenum and Phosphorus on Copper Metabolism—The procedures developed for the use of Cu^{64} have already been described (10). A study with Cu^{64}, similar to the one outlined above for P^{32}, was undertaken with normal mature piebald rats. Table IV shows the effect of

TABLE IV

Effect of Molybdenum and Phosphorus on Accumulation of Ingested Copper in Tissues of Rat

The results are expressed as micrograms of labeled copper per gm. of fresh tissue, based on a 10 mg. dose.

Dosage	Liver		Blood	Lung	Spleen	Kidney	Muscle	Hide
7.3 mg. Cu^{64}	46		15	2	5	13	0.5	0.2
	±6	(2.5)*	±1	±0.3	±3	±3	±0.3	±0.03
Same + 40 mg. Mo	18		11	4	4	8	0.6	0.3
	±5	(1.2)	±2	±2	±2	±1	±0.4	±0.04
" + 23 " P	9		10	3	3	5	0.7	0.4
	±5	(0.5)	±3	±2	±1	±1	±0.6	±0.3
5.4 mg. Cu^{64} + 24 mg.	7		13	3	3	6	0.7	0.4
Mo + 23 mg. P	±0.8	(0.5)	±2	±0.4	±0.8	±1	±0.1	±0.2

* The mean value plus-minus the mean deviation; the values in parentheses represent the percentage of the dose in the whole organ. There were four rats in each group.

simultaneously ingested molybdenum and phosphorus upon the tissue accumulation of labeled copper; all animals were sacrificed 18 hours after dosage. It is evident that molybdenum and phosphorus decreased the liver accumulation of copper by 2½- and 5-fold, respectively; with the other tissues it should be noted that except for the kidney there was little or no significant reduction.

The effect of molybdenum on copper storage in the liver of the bovine has also been demonstrated by use of the radioisotope. Two calves, 3½ and 5 months old, which had been on a high molybdenum ration previous to the experiment accumulated 0.13 and 0.82 per cent of orally administered copper in the liver after 42 hours; in a parallel study three calves receiving no supplemental molybdenum showed values of 3.8, 3.8, and 4.6 per cent. Numerous analyses of livers of mature cattle maintained

molybdenum as Na_2MoO_4. Rats of Group C were fed 22 mg. of molybdenum as Na_2MoO_4 daily for 3 days preceding administration of the labeled phosphorus. All the rats were sacrificed at 24 hours after dosage.

Table III shows the concentrations in selected tissues calculated to a common basis for comparative purposes. A comparison of Groups 1 and 2 shows that the simultaneous administration of copper had little, if any, effect on the phosphorus accumulation. However, the values from Group 3 are in each case slightly but significantly higher than those of Group 1,

TABLE III

Effect of Molybdenum and Copper on Accumulation of Ingested Phosphorus in Tissues of Rat

The results are expressed as micrograms $\times 10^1$ of labeled phosphorus per gm. of fresh tissue based on a $1\,\gamma$ dose per 100 gm. of body weight.

Group No.	No. of rats	Age	Treatment	Blood	Lung	Spleen	Liver	Kidney	Muscle	Bone	Hide
1	10	Mature	P³²	4	11	13	33	20	5	20	3
				±1*	±2	±6	±10	±5	±1	±6	±1
2	10	"	" + Cu	4	13	19	44	23	5	22	2
				±2	±6	±8	±17	±8	±2	±6	±0.6
3	9	"	" + Mo	6	18	24	48	32	7	46	6
				±2	±7	±8	±6	±6	±3	±15	±2
4	6	"	" + Mo + Cu	3	8	18	29	14	5	13	3
				±1	±3	±6	±7	±5	±3	±3	±0.3
A	12	Young	"	13	30	63	88	66	20	260	11
				±4	±9	±17	±18	±18	±5	±60	±3
B	11	"	" + Mo + Cu	9	24	55	68	50	11	133	7
				±2	±7	±8	±17	±8	±4	±35	±3
C	14	"	Mo + P³²†	9	28	47	53	51	17	199	8
				±2	±5	±15	±11	±11	±4	±38	±1

Labeled P dosage, $2.14\,\gamma$; additional Cu, 5.18 mg.; additional Mo, 34.3 mg.

* The mean value plus-minus the mean deviation.

† Rats given 22 mg. of Mo per day for 3 days preceding P³² dosage

which indicates that the simultaneous administration of molybdenum apparently increased the phosphorus uptake. The values from Group 4 indicate that the combination of copper and molybdenum had a tendency to reduce the phosphorus accumulation. A comparison of Groups 1 and A shows the effect of age; the younger rats accumulated more phosphorus and, as would be expected, the difference was greatest in the case of the bone. Group B shows again the reduction in phosphorus uptake caused by simultaneous administration of molybdenum and copper. The values from Group C indicate that when molybdenum is fed sometime previously

When 40 mg. of $Mo^{99}O_3$ were administered by stomach tube to five rats, the urinary-fecal ratio was 1.6 ± 0.6; however, when 23 mg. of phosphorus as Na_2HPO_4 were simultaneously given, the urinary-fecal ratio in four rats increased to 9.0 ± 3. This indicated that phosphorus increased the absorption of the molybdenum, but that retention was so low that no differences were found in tissue accumulations even though the animals

<div align="center">

TABLE II

Comparison of Phosphorus and Molybdenum Accumulation in Selected Tissues of Bovine

</div>

Concentration of vertebra set at 1000.

	Jugular injection		Oral administration	
	Phosphorus	Molybdenum	Phosphorus	Molybdenum
Vertebra	1,000	1000	1000	1000
Long bone (femur)		623	694	1790
Rib bone	970	1340		2330
Thyroid	535	204	27	0
Thymus	348	182	430	109
Adrenals	4,018	1550	818	815
Brain	76	72	100	0
Eye	108	1200	33	334
Intestinal lymph glands	1,270	1050	845	1690
Lung	1,030	500	510	255
Kidney	2,900	1200	780	976
Abomasum	545	175	240	195
Small intestine	350	404	451	402
Large "	580	334	405	192
Pancreas	2,060	302	759	143
Spleen	10,060	342	686	865
Liver	5,350	1210	963	1430
Tenderloin muscle	580	78	311	0
Cartilage	372	278	123	530
Red bone marrow	1,580	555	1240	431
White bone marrow	108	58	20	242

were sacrificed after 18 to 20 hours. Most of the urinary excretion occurred within 24 hours after dosage.

Effect of Molybdenum and Copper on Phosphorus Metabolism—Mature and 8 to 9 week piebald rats on a regular stock ration were used to determine the effect of molybdenum and copper on the absorption and tissue distribution of labeled phosphorus. In each case, except Group C, 1 ml. of solution containing the activity plus the additional elements was administered to the fasting rat by stomach tube. The amounts used were 2.14 γ of P^{32} as Na_2HPO_4, 5.18 mg. of copper as $CuSO_4$, and 34.3 mg. of

TABLE I—*Concluded*

Mode of administration Age of animal, *mos.* Weight of animal, *lbs.* Actual dosage, *gm. of MoO₃* Sacrificed after, *days*	Oral 14 380 29 12		Jugular injection 14 380 1.9 4	
	MoO₃ per 100 gm. fresh weight per 100 mg. dosage	Per cent of dose in whole tissue	MoO₃ per 100 gm. fresh weight per 100 mg. dosage	Per cent of dose in whole tissue
	γ		γ	
Small intestine contents .	0.34	0.0074	7.9	0.30
Large " mucosa	2.16⎱	0.013	11.6⎱	0.16
" " muscular	0.39⎰		7.4⎰	
" " contents .	0.61	0.021	22.8	0.52
Pancreas	0.54	0.00087	6.6	0.012
Spleen..	3.29	0.012	7.5	0.032
Liver . . .	5.47	0.12	26.5	0.69
Gallbladder			5.2	0.0012
Bile . .	0.34	0.00009	1.6	0.00082
Tenderloin muscle	*		1.7	
Gastrocnemius muscle	0.20		3.8	
Ligament .	0.19		5.7	
Cartilage	2.0		6.1	
Long bone (femur)	6.8		19.5	
Rib bone	8.9		29.4	
Vertebra	3.8		21.9	
Periosteum .	*		9.6	
Red bone marrow	1.65		12.2	
White bone marrow	0.92		1.3	
Teeth .	4.37		15.6	
Hide .	0.92	0.14	8.9	1.41
Collected feces .		34.2		11.0
" urine		45.2		36.7

* No radioactivity detected in sample.

has been set at 1000. The phosphorus values were obtained with P^{32} from animals sacrificed 1 day after injection and 2 days after oral administration.

Effect of Phosphorus and Copper on Molybdenum Metabolism—Numerous experiments have been run in which Mo^{99} was fed to rats alone, with phosphorus, with copper, and with both to determine whether the accumulation in the tissues was changed. Significant differences were not observed, which agreed with Neilands *et al.* (7), who reported that the simultaneous administration of copper did not affect the molybdenum distribution.

TABLE I

Tissue Distribution of Labeled Molybdenum Administered to Cattle

	Oral		Jugular injection	
Mode of administration				
Age of animal, *mos.*	14		14	
Weight of animal, *lbs.*	380		380	
Actual dosage, *gm. of MoO₃*	7 0		1.9	
Sacrificed after, *days*	12		4	
	MoO₃ per 100 gm. fresh weight per 100 mg. dosage	Per cent of dose in whole tissue	MoO₃ per 100 gm. fresh weight per 100 mg. dosage	Per cent of dose in whole tissue
	γ		γ	
Pituitary	*	0	13.8	0.000089
Thyroid	*	0	4.5	0.00047
Thymus	0.42	0.00035	4.0	0.012
Adrenals	3.2	0.00041	34.1	0.00037
Penis	0.63	0.0013	9.9	0.02
Salivary glands	0.38	0.00050	5.6	
Parotid glands	0.81	0.00085	6.6	
Cerebrum	*	0	2.1	0.00077
Cerebellum	*	0	1.0	0.0027
Spinal cord			1.4	
Eye	1.28	0.00082	26.5	0.017
Intestinal lymph glands	6.45		23.2	
Heart	*	0	3.6	0.033
Blood	0.50	0.066	11.1	1.5
Aorta	0.31	0.00024	5.7	0.0075
Lung	0.97	0.016	11.0	0.17
Trachea	0.16	0.00036	5.8	0.0097
Kidney	3.73	0.017	26.3	0.13
Bladder	6.1	0.0052	7.1	0.0054
" urine	0.86	0.00061	38.8	0.062
Tongue	0.24	0.0013	4.9	0.028
Esophagus	0.40	0.00090	4.8	0.012
Fundus abomasum mucosa	* ⎫		5.2 ⎫	
" " muscular	0.46 ⎬ 0.0031		3.3 ⎬ 0.037	
Pyloric abomasum mucosa	* ⎪		6.2 ⎪	
" " muscular	0.35 ⎭		4.4 ⎭	
Abomasum contents	0.59	0.0046	11.4	0.21
Reticulum	0.40	0.0019	7.6	0.036
Omasum	0.29	0.0033	6.1	0.074
" contents	0.47	0.010	1.7	0.27
Rumen	0.39	0.012	11.2	0.38
" and reticulum contents	0.28	0.056	7.1	1.16
Duodenum, mucosa	1.65 ⎫		7.8 ⎫	
" muscular	0.50 ⎪		3.4 ⎪	
Jejunum, mucosa	2.25 ⎬ 0.037		10.9 ⎬ 0.30	
" muscular	1.75 ⎪		6.5 ⎪	
Ileum, mucosa	1.21 ⎪		13.3 ⎪	
" muscular	1.18 ⎭		5.9 ⎭	

Throughout the experiment the animals in Group 1 were of normal appearance and behavior. The animals in Group 2 were normal during the first part of the trial, but a lightening of the coat of hair near the end of the experiment indicated the development of copper deficiency. The animals in Group 3 showed a retarded skeletal growth with poor calcification; they developed diarrhea, a rough coat of hair, and in severe cases developed an excessive lacrimation of the eyes. It is recognized that the basal ration was not completely adequate, but the experiment does, nevertheless, emphasize the effect of nutritional status on molybdenum toxicity. This indicates that molybdenum toxicity must be considered in terms of the copper level of the diet, or conversely, a copper deficiency must take into account any molybdenum present. It has also been observed that molybdenum toxicity is much less pronounced in the mature rat.

Molybdenum Metabolism—Tracer studies were undertaken with radioactive Mo^{99}. This isotope was obtained as a pile-produced irradiation unit consisting of 10 gm. of MoO_3 with an initial activity of about 40 mc. The material as received was transferred to a 400 ml. beaker and 35 ml. of 14.3 per cent NaOH were added. The mixture was stirred mechanically until a clear solution was obtained, which was then diluted to a convenient volume with distilled water to give a pH near 7. No significant radioactive contaminants were expected in this unit on the basis of the analysis of the target material, and the observed half life agreed well with the established value of 67 hours. The administration to the animals and the collection, handling, and assay of the samples were the same as described for copper and cobalt (8, 9).

Since the ruminant is apparently more sensitive to copper-molybdenum imbalance than are the species with single stomachs, cattle were also used for experimentation. Table I shows the tissue distribution of Mo^{99} injected and orally administered to 14 month-old steers. It was apparent from the excretion values that considerable absorption of ingested molybdenum from the tract occurred. There was general distribution of the administered molybdenum in both cases; however, retention in the various tissues was low. In another experiment the percentages of the ingested dose found in the blood at 16, 41, 64, and 95 hours were 0.6, 2.9, 2.6, and 1.5, respectively; the corresponding values for the liver as determined by biopsy for the first three intervals were 0.26, 0.51, and 0.42. This indicated rather rapid absorption with a peak at 41 hours after dosage. Of particular interest was the observation of high selective accumulations in the adrenals, bone, liver, eye, intestinal lymph glands, and kidney.

Table II shows the definite similarity between phosphorus and molybdenum accumulation in the tissues of the bovine despite the differences in time of sacrifice. For comparison, the concentration in the vertebra

the fatalities were much less at a 120 to 600 mg. level. Intraperitoneal injection experiments indicated a high mortality rate for guinea pigs injected with soluble molybdenum compounds in amounts of from 400 to 800 mg. per kilo. Neilands *et al.* (7) have recently reported on the toxicity of sodium molybdate fed in a purified ration to the 21 day-old rat; a level of 5000 p.p.m. of molybdenum resulted in death after the 1st week, whereas levels of 1000 and 500 p.p.m. permitted a greatly reduced growth rate. The toxicity of a diet containing 400 p.p.m. was largely overcome by feeding an additional 20 p.p.m. of copper to a diet already containing 77.3 p.p.m.
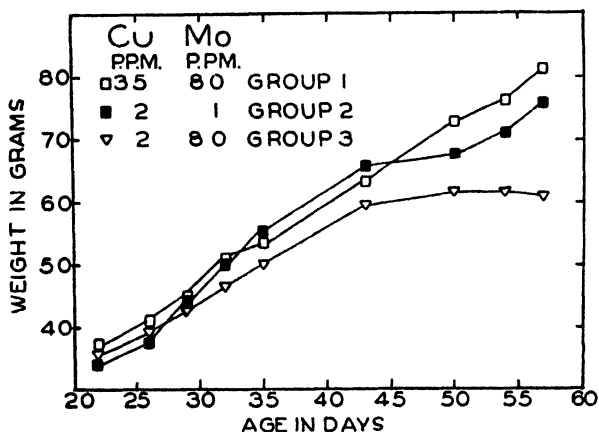
Fig. 1. Effect of copper and molybdenum on rat growth

EXPERIMENTAL

In order to study the effect of low molybdenum levels when the ration was also low in copper, a number of 21 day-old piebald rats were placed on the following basal ration: 3418 gm. of whole milk powder, 3381 gm. of sucrose, 34 gm. of sodium chloride, 0.136 gm. of ferrous sulfate, 0.06 gm. of manganous sulfate, and 0.023 gm. of thiamine hydrochloride. The copper content, by analysis, was 2 p.p.m. and the molybdenum about 1 p.p.m. Fig. 1 shows the growth retardation caused by a level of 80 p.p.m. of molybdenum in this ration and effective compensation by an increase to 35 p.p.m. of copper. At 57 days the fifteen rats in Group 1 (35 p.p.m. of copper, 80 p.p.m. of molybdenum) showed an average gain of 44 ± 4 gm., while the sixteen rats in Group 2 (2 p.p.m. of copper, 1 p.p.m. of molybdenum) averaged 42 ± 8 gm., and thirteen rats out of an original fifteen in Group 3 (2 p.p.m. of copper, 80 p.p.m. of molybdenum) averaged 24 ± 13 gm. At 67 days, eight rats out of fifteen in Group 3 had died, while three had been sacrificed earlier for observation.

MOLYBDENUM METABOLISM AND INTERRELATIONSHIPS
WITH COPPER AND PHOSPHORUS*

By C. L. COMAR,† LEON SINGER, and GEORGE K. DAVIS

(From the Florida Agricultural Experiment Station, Gainesville)

In 1938 it was discovered that naturally occurring molybdenum in certain pastures in the United Kingdom was responsible for a severe disease of cattle and sheep (1). The unhealthy pastures were characterized by a molybdenum content of 20 to 100 parts per million on the dry basis, as compared with less than 5 p.p.m. in the normal pasture. The experimental production of the disease by administration of sodium molybdate to healthy cattle confirmed the toxic action of this element. The literature on molybdenum in relation to the health of farm animals has been reviewed to 1944 by Russell (2). Potentially toxic levels of molybdenum have since been reported in parts of Florida and California (3, 4).[1]

A most satisfactory feature of the molybdenum problem has been the finding that supplemental copper will apparently cure or prevent the condition. The symptoms of this disease are remarkably similar to those of copper deficiency,[1] and the therapeutic action of copper indicates a relationship between these two elements which is as yet unexplained. An outstanding symptom of copper deficiency or copper-molybdenum imbalance is the interference with normal bone development. This observation, in addition to the findings that alkaline blood phosphatase values in cattle were affected by the copper status of the animal (5), and that molybdenum metabolism was similar to that of phosphorus, has indicated that phosphorus may also be involved in the copper-molybdenum antagonism.

Molybdenum Toxicity—Fairhall *et al.* (6) have presented a comprehensive compilation of the literature on molybdenum up to 1945 and described an experimental study of its toxicity. It was found that soluble molybdenum compounds when fed to rats and guinea pigs in large doses of from 1200 to 6000 mg. per kilo of body weight invariably proved fatal;

2. Iron and oxyhemoglobin values were shown to vary consistently. Approximately 1.6 per cent of non-hemoglobin iron was found to be present.

3. A linear relationship was established for oxyhemoglobin and acid hematin at 410 mμ. The standard deviation from regression was 0.12 gm. per cent of hemoglobin, even when values from hemoglobin solutions were included in the data.

4. Two linear expressions were evident for oxyhemoglobin and acid hematin densities read at 450 mμ. One was for blood from immature and anemic birds and the other was for blood from adult birds and hemoglobin solutions from which most of the nucleoprotein had been removed.

5. The nucleoprotein of avian blood appears to interfere with acid hematin density (log I_0/I) at 450 mμ but does not do so appreciably at 410 mμ. The reasons for this are discussed.

6. There was only one linear expression for oxyhemoglobin and alkaline hematin at 450 mμ. The standard deviation from regression was less at 450 than at 400 mμ for alkaline hematin.

7. The manometric method appears to be the most accurate method for hemoglobin analyses of avian blood.

BIBLIOGRAPHY

1. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
2. Klein, D. E., Hall, J. L., and King, H. H., *J. Biol. Chem.*, **105**, 753 (1934).
3. Schultze, M. O., and Elvehjem, C. A., *J. Biol. Chem.*, **105**, 253 (1934).
4. Winegarden, H. M., and Borsook, H., *J. Cell. and Comp. Physiol.*, **3**, 437 (1933).
5. Bankowski, R. A., *Am. J. Vet. Res.*, **3**, 373 (1942).
6. Rostorfer, H. H., and Rigdon, R. H., *Am. J. Physiol.*, **146**, 222 (1946).
7. Rostorfer, H. H., and Rigdon, R. H., *Am. J. Clin. Path.*, **16**, 518 (1946).
8. King, E. J., Gilchrist, M., and Delory, G. E., *Lancet*, **1**, 239 (1944).
9. Van Slyke, D. D., Hiller, A., Weisiger, J. R., and Cruz, W. O., *J. Biol. Chem.*, **166**, 121 (1946).
10. Sumner, J. B., and Somers, G. F., Laboratory experiments in biological chemistry, New York, 110 (1944).
11. Saywell, L. G., and Cunningham, B. B., *Ind. and Eng. Chem., Anal. Ed.*, **9**, 67 (1937).
12. Bernhart, F. W., and Skeggs, L., *J. Biol. Chem.*, **147**, 19 (1943).
13. Gibson, Q. H., and Harrison, D. C., *Biochem. J.*, **40**, 247 (1946).
14. Nicloux, M., and Fontes, G., *Bull. Soc. chim. biol.*, **6**, 728 (1924).

determining "total hemoglobin," in which $Na_4S_2O_4$ is used to reduce the Fe^{+++} of inactive hemoglobin to Fe^{++} of active hemoglobin, may not be applied to similar analysis of avian blood because of the difficulty of equilibrating laked avian blood in the extraction chamber. This method was not investigated, but samples of avian blood were analyzed after equilibrating in tonometers, and the values were compared with values for samples equilibrated in the extraction chamber during which laking took place, the latter values being always lower when both were properly corrected by the respective C corrections. It might be possible to equilibrate hemoglobin solutions from which the nuclei have been removed in the extraction chamber and obtain comparable results with tonometer-equilibrated samples. However, it was thought that total iron analysis was a better approach, since the validity of the gasometric method was in doubt.

There is rather close agreement between values obtained by manometric analysis and acid hematin density (log I_0/I) at 410 mμ. The sigma from regression at 410 mμ was small and acid hematin prepared by the method of Schultze and Elvehjem and read in the spectrophotometer at this wavelength varied linearly with the oxyhemoglobin. The regression failed to pass through the origin which can be interpreted to be caused by the presence of some substance which increases the density reading. However, the acid hematin method read at 410 mμ would appear to give sufficiently accurate estimates of avian hemoglobin content. This is true for alkaline hematin read at 450 mμ when the samples are heated in a boiling water bath for 4 minutes.

Cyanhematin and pyridine hemochromogen methods have been investigated by the author as possible approaches to hemoglobin determination for avian blood. The cyanhematin method is useless because of the appearance of a flocculent precipitate and much of the cyanhematin is adsorbed on the precipitate or otherwise interfered with, so that the absorption values are quite low in comparison with cyanhematin values for dog blood with similar oxygen capacity. The pyridine hemochromogen gives unpredictable values for avian blood because of the rapid fading of the color presumably due to reoxidation.

The Van Slyke manometric method appears to be the most accurate means of hemoglobin analysis for avian blood, according to these experiments, if the proper precautions are taken in equilibration to avoid laking or utilization of oxygen by the red blood cells after the sample has been equilibrated.

SUMMARY

1. Blood from normal immature and adult ducks, anemic adult ducks, and samples of hemoglobin solution have been analyzed for total iron, oxyhemoglobin, and acid and alkaline hematin.

comes apparent. Some substance, probably nucleoprotein, interferes with the acid hematin density at 450 mμ, possibly by producing a Tyndall effect.

The variation in acid hematin density values between duplicate samples was significantly less at 410 mμ than between duplicate determinations of acid hematin at 450 mμ.

Alkaline hematin values for samples from normal immature, adult, anemic ducks and hemoglobin solution are treated in a similar manner in Fig. 2. Line D represents the data at 400 mμ and Line E at 450 mμ. There is only one true linear relation at either wave-length. The standard deviation of the data from Line D is 0.022 log unit or 0.67 gm. of hemoglobin per 100 ml. of blood and the standard deviation from regression for Line E is 0.008 log unit or 0.39 gm. of hemoglobin. There is greater deviation in the data for alkaline hematin at 400 mμ than for those at 450 mμ. However, the data in Table II for alkaline hematin from hemoglobin solutions and whole blood indicate some variation, since the average value of the density at 450 mμ for alkaline hematin prepared from whole blood is 0.142 in comparison to 0.133 for hemoglobin solutions.

<center>DISCUSSION</center>

The lack of agreement between hemoglobin values from avian blood determined by gasometric analysis and by hemoglobin estimated to be present by colorimetric methods based on the density of acid hematin has led various investigators to question the accuracy of the Van Slyke manometric method for oxyhemoglobin determinations of avian blood. The experiments described above would indicate that there is a considerable variation in the density readings of acid hematin, depending upon whether these readings are made at 410 or at 450 mμ. The standard deviation at 450 mμ was several times that for the data at 410 mμ. Acid hematin density values are usually read at 450 mμ and some standards such as the Newcomer plate are based on absorption at 520 mμ. Thus, it would appear that these earlier determinations by Schultze and Elvehjem, Bankowski, and Rostorfer and Rigdon were made at a wave-length at which the density of acid hematin is quite variable. The lack of agreement mentioned above appears to be caused by the variation in acid hematin values and not by an unpredictable error in the manometric analyses.

Furthermore, the iron found by analysis for duck blood varied from the iron calculated from the oxyhemoglobin in a predictable manner and within the limits reported for mammalian blood, for which the manometric method has been shown to be the most accurate of all analytical approaches.

There seems to be a small amount of non-hemoglobin iron present in duck blood, but whether the amount is the same or actually greater than that found for man or other mammals is not clearly demonstrated by the experiments described here. The method of Nicloux and Fontes (14) for

Line C was plotted by the method of least squares from the data obtained from adult ducks and hemoglobin solutions. Its slope is 0.00872 and it intercepts the y axis at 0.004 log unit. The standard deviation from regression is 0.012 log unit or 1.25 mg. per cent of iron, which is equal to 0.37 gm. of hemoglobin.

When the two regressions are analyzed statistically for significance, the t value is 2.29 and the probability is about 0.01. These two relationships reflect the differences in the ratios (the density at 410 mμ divided by the density at 450 mμ) between acid hematin prepared from blood of adult
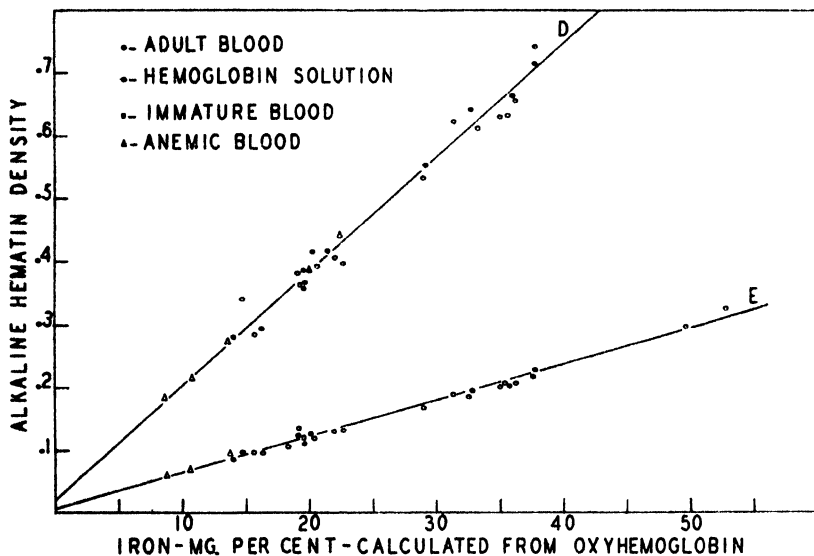
Fig. 2. Linear relationships for alkaline hematin and oxyhemoglobin iron. Line D, regression for alkaline hematin density read at 400 mμ, and Line E, regression for alkaline hematin at 450 mμ.

ducks and solutions of hemoglobin on the one hand and that prepared from young ducklings or anemic ducks on the other. This is in agreement with the results reported by Rostorfer and Rigdon for ducklings and adult ducks.

That there are two regressions for these data becomes still more evident if a common regression (not shown in Fig. 1) is plotted for the data represented by Lines B and C. The standard deviation from this assumed regression is 2.08 mg. per cent of iron or 0.62 gm. of hemoglobin per 100 ml. of blood. This is over 5 times the standard deviation for the data for Line A. The difficulty in the use of acid hematin analysis read at 450 mμ as a means of determining the amount of hemoglobin in avian blood now be-

analyses were carried out on the assumption that the values (oxyhemoglobin iron) plotted on the *x* axis were determined without error. The standard deviation from regression is equal to 0.5 mg. per cent of iron or approximately 0.12 gm. of hemoglobin. The values for hemoglobin solution and anemic, immature, and adult blood samples do not vary significantly from those shown by Line A. There appears to be only one linear relationship between acid hematin values at 410 mμ and hemoglobin values determined gasometrically.
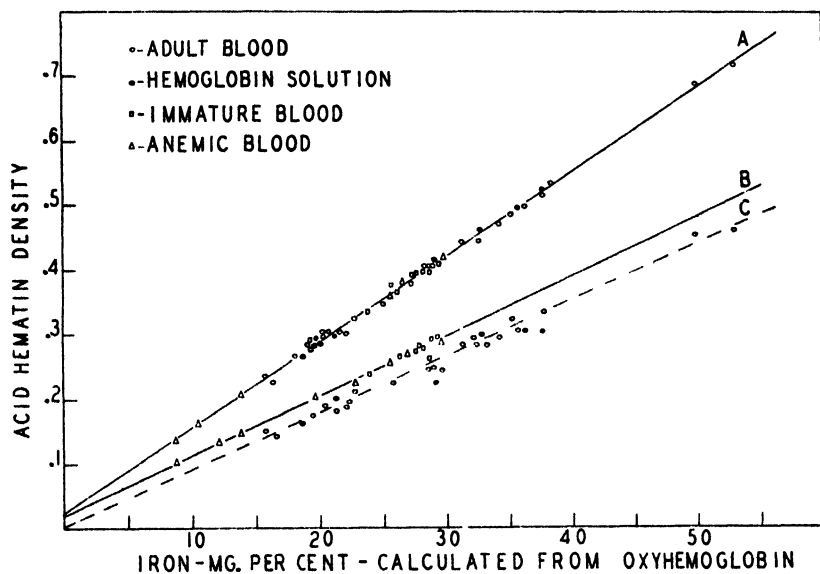
Fig. 1. Linear relationships between acid hematin density and oxyhemoglobin iron calculated from values obtained by the Van Slyke analysis. Line A, regression when density values were read at 410 mμ; Line B, regression for blood obtained from young and anemic ducks when density readings were made at 450 mμ; Line C, regression for blood from adult ducks and hemoglobin solution when density values were determined at 450 mμ.

The relationships for acid hematin densities at 450 mμ are shown by Lines B and C. The results of all samples analyzed at 450 mμ and plotted on Lines B and C were also analyzed at 410 mμ and plotted on Line A; thus it is evident that the deviation represented by Lines B and C at 450 mμ is not present at 410 mμ. Line B shows the regression for acid hematin and oxyhemoglobin prepared from young and anemic duck blood. It was fitted to the data by the method of least squares. The slope of Line B is 0.00929 and the line intercepts the *y* axis at 0.019 log unit. The standard deviation from regression is 0.009 log unit or 0.2 mg. per cent of iron.

lings. The hemoglobin solutions were prepared as described above and were relatively free of nucleoprotein. It will be noted that the average iron values for the whole blood samples and solutions, whether calculated from the oxyhemoglobin obtained by gasometric analysis or determined analytically, are not significantly different; *i.e.*, the same amounts of hemoglobin were present in the samples of whole blood and solutions.

If there were errors in gasometric analysis caused by incomplete laking of the cells or by retention of gas in the gelatinous nucleoprotein in the extraction chamber, there should be a considerable difference between the values obtained for whole blood and solution as determined by the gasometric method, since there are no cells in the solution and no nucleoprotein to form a gel in the extraction chamber. However, the values for whole blood and solution agree.

The densities (log I_0/I) for acid hematin prepared from whole blood and from hemoglobin solution were determined at both 410 and 450 mμ. The ratios (density at 410 mμ divided by density at 450 mμ) are significantly different for whole blood and solution. The average ratio for whole blood is 1.48 and for the hemoglobin solution 1.64. The average density at 410 mμ is higher for the hemoglobin solution and the average density at 450 mμ is higher for the whole blood. The absorption curve for whole blood is not as steep as that for the hemoglobin solution. The difference in slope of the absorption curves for alkaline hematin prepared from whole blood and from hemoglobin solution is not so apparent, although there is some increase in the slope of the curve for alkaline hematin prepared from the solution of hemoglobin. The hemoglobin solution differs from whole blood mainly because of the greatly reduced nucleoprotein content.

Iron calculated from oxyhemoglobin values determined by gasometric analysis and the iron values obtained by analysis of whole blood samples and hemoglobin solution are in close agreement; thus the variation between whole blood and solution appears to be in the density reading of acid and alkaline hematin. The lowest ratios (density at 410 mμ divided by density at 450 mμ) are shown for the whole blood of both the anemic adult and the duckling.

The variation of density values for acid hematin solutions read at 450 mμ is considerably greater than at 410 mμ. This is illustrated by Fig. 1. The results of acid hematin determinations at 410 mμ for whole blood samples of normal young and adult ducks and anemic adults and hemoglobin solutions from the same sources are plotted against mg. per cent of iron calculated from oxyhemoglobin determined by the Van Slyke manometric analysis. Line A was fitted to these data by the method of least squares. The slope of the line is 0.0133 and the line intercepts the y axis at 0.024 log unit; thus it fails to pass through the origin. Statistical

The amount of extra iron reported here is comparable to that reported to be present in mammalian blood by Gibson and Harrison (13). The highest percentage of non-hemoglobin iron in Table I is 6.1 per cent and this is nearly 4 times the standard deviation for the adult group. It does not seem probable that there could be as much as 12 per cent or even 5 per cent

TABLE II

Comparison of Iron in Blood and Hemoglobin Solution

Iron calculated from oxyhemoglobin and iron found analytically with acid hematin (density at 410 and 450 mμ), the ratio (density at 410 mμ divided by density at 450 mμ), and alkaline hematin (density at 400 and 450 mμ) for samples of whole blood and of hemoglobin solution with nuclei removed prepared from whole blood samples.

Sample No.	Iron mg per cent		Acid Hb			Alkaline Hb	
	HbO₂ Fe	Analytic Fe	410 mμ	450 mμ	Ratio	400 mμ	450 mμ
Whole blood							
1	33.27	33.74	0.431	0.286	1.51	0.611	0.188
2	28.99	29.75	0.408	0.250	1.63	0.539	0.167
3	20.55	21.40	0.304	0.201	1.51	0.394	0.120
4	19.65	19.53	0.285	0.204	1.40	0.385	0.120
5	19.54	19.75	0.245	0.178	1.37	0.362	0.115
Average...	24.40	24.83	0 334	0.224	1.48	0.458	0.142
Extra Fe, %	1.7						
Hemoglobin solution							
	32.97	33.74	0.488	0.300	1.62	0.642	0.195
	29.27	29.00	0.414	0.225	1.84	0.554	0.143
	21.38	22.50	0.297	0.183	1.62	0.416	0.112
	19.72	19.50	0.292	0.193	1.51	0.368	0.110
	18.53	18.75	0.266	0.165	1.60	0.336	0.105
Average ..	24.37	24.70	0.351	0.213	1.64	0.463	0.133
Extra Fe, %	1.3						

more iron present than that found in combination with hemoglobin. Some small percentage of non-hemoglobin iron appears to be present and this percentage is comparable to that found for mammalian blood by the use of similar methods.

The results of analysis of whole blood and hemoglobin solutions are recorded in Table II. Samples 1, 2, and 3 were obtained from normal adult ducks, Sample 4 from an anemic adult, and Sample 5 from 7 day-old duck-

blood or hemoglobin solution present. The 1 ml. Van Slyke sample was removed by gravity and placed in the apparatus within 1 minute after equilibration ceased. Rostorfer and Rigdon (6) demonstrated no appreciable error in this technique by comparing duplicate samples equilibrated at 0° and at 39°.

TABLE I

Comparison of Iron Values Found by Calculation from Oxyhemoglobin and by Analysis in Blood Samples Obtained from Normal Young and Adult Ducks and from Anemic Ducks

The values are measured in mg. per cent.

Adult		Young		Anemic	
HbO₂ Fe	Analytic Fe	HbO₂ Fe	Analytic Fe	HbO₂ Fe	Analytic Fe
30.39	31.12	28.45	28.50	29.30	29.00
31.14	30.87	19.72	20.00	26.54	27.00
28.79	29.06	25.56	26.25	12.37	13.30
18.28	18.25	19.51	19.75	19.67	19.53
20.91	20.77	13.86	13.75	13.90	14.75
33.49	34.00	27.53	28.00		
		27.50	28.00		
24.54	25.50	28.90	28.57		
21.48	22.50	27.40	28.00		
29.92	31.75	26.18	26.25		
28.99	29.75	23.80	23.75		
33.27	33.74				
20.55	21.40				
Average..26.81	27.38	24.40	24.62	20.36	20.72
Extra Fe, %.. ... ..2.1		0.9		1.8	

Results

In Table I iron values obtained by direct analysis of various samples of blood are compared to the iron values calculated from oxyhemoglobin obtained by the Van Slyke manometric method. The hemoglobin iron was calculated by the use of the factor 0.340 per cent. Bernhart and Skeggs (12) redetermined the percentage of iron in crystalline human hemoglobin and reported it to be 0.340 per cent; therefore, this factor was used instead of the value 0.335 per cent in calculating the amount of hemoglobin iron. The average amount of non-hemoglobin iron is about 1.6 per cent. If the factor 0.335 per cent is used instead of 0.340, the non-hemoglobin iron is about 3 per cent. The difference in percentages of non-hemoglobin iron present in the young, adult, and anemic birds is not statistically significant.

heart puncture until a marked anemia was established, as shown by the low red blood cell count and the presence of young erythrocytes and erythroblasts in the peripheral circulation.

Blood for analysis was always obtained by heart puncture. Coagulation was prevented by adding 5 ml. of 2 per cent citrate in saline to each 25 ml. of blood. If the blood was withdrawn rapidly, there was usually no evidence of clotting. Samples were strained through cotton to remove any minute clots which might have formed. All blood samples were stored at 5° until used and were removed for analysis within 3 hours after the heart puncture.

Hemoglobin solutions were obtained by centrifuging 10 ml. of blood and then carefully pipetting off the supernatant plasma. This was carefully measured and replaced by an exactly similar amount of distilled water, whereupon the sample was vigorously shaken and centrifuged for 10 minutes at maximum speed. The nuclei were precipitated, leaving the hemoglobin volume relationship the same as in the original sample of blood. There was no evidence of incomplete laking of the cells. The hemoglobin solutions were clear and relatively free of nuclear material.

Iron analyses were carried out according to the method described by Sumner and Somers (10), which is essentially the phenanthroline colorimetric method of Saywell and Cunningham (11). Samples were analyzed in quadruplicate by use of the Evelyn photometer and compared to a standard curve obtained by analyzing analytical iron wire.

Acid hematin determinations were made according to the technique of Schultze and Elvehjem (3) for chicken blood, except that the Beckman quartz spectrophotometer was used instead of the modified Newcomer procedure described by these authors. Since the object of these experiments was to investigate the correlation of the per cent of absorption of transmitted light by acid hematin with the amount of hemoglobin obtained by the Van Slyke analyses, the samples were not corrected for the volume change caused by the addition of 0.12 ml. of concentrated hydrochloric acid. The samples were read at 410 and 450 $m\mu$. Absorption curves for acid hematin prepared from duck blood were also obtained by use of the spectrophotometer.

Alkaline hematin was prepared by laking 0.01 ml. of blood in 5.00 ml. of 0.1 N NaOH. This solution was allowed to stand for some convenient time and then heated in boiling water for 4 minutes, cooled, and the per cent absorption of the transmitted light read at 400 and at 450 $m\mu$ by use of the spectrophotometer.

Oxyhemoglobin was determined by the Van Slyke manometric apparatus. The procedure described by Van Slyke and Neill (1) was followed. All samples were equilibrated in air in a 250 ml. tonometer with 5 ml. of

tween duplicate samples to be quite small in comparison to the variation between duplicate samples of acid hematin. Furthermore, oxygen utilization by the red cells as determined by the Warburg technique agreed with values found by determining the oxygen capacity of equilibrated cells confined to space for a given time and compared with the oxygen capacity of freshly equilibrated samples of the same blood. Also, oxygen dissociation curves for duck blood were expressed by Hill's formula

$$y = \frac{Kx^n}{100 + Kx^n}$$

These discrepancies have been variously explained as being due to differences in percentages of iron in avian hemoglobin, the presence of non-hemoglobin iron, errors in the Van Slyke analysis possibly caused by incomplete hemolysis and trapping of gas in the gelatinous nucleoprotein in the extraction chamber, and by the possibility of error in the acid hematin determinations.

Since the oxygen dissociation curve for avian blood is somewhat unique and, therefore, of interest, it occurred to the author that the whole problem should be reinvestigated to find the source of error or errors in these determinations. King et al. (8) have stated that the Van Slyke method is the most accurate of all methods for determination of hemoglobin in mammalian blood. In a recent study of the relationship of "total" and "active" hemoglobin, Van Slyke et al. (9) have demonstrated the presence of about only 1 per cent "inactive" hemoglobin in freshly drawn human blood. These authors state that hemoglobin values obtained by either O_2 or CO capacity were near 100 per cent of the "total" hemoglobin determined by the use of reducing agents, when samples were allowed to stand an hour or more before being analyzed. If the Van Slyke manometric method proved to be valid for avian blood, the oxygen dissociation curve of avian blood might have to be considered to represent a difference in the behavior of avian hemoglobin in comparison to mammalian hemoglobin.

The results given below were obtained by carrying out analyses for iron, acid and alkaline hematin, and oxyhemoglobin on blood samples obtained from normal young and adult ducks, and from anemic adults. The blood was variously diluted with 0.9 per cent saline or concentrated by removing plasma to obtain a range.

Methods and Materials

Ducklings and mature normal white Pekin ducks were used in the experiments described. All ducklings were fed Purina Startina for about 2 weeks and all ducks were fed Purina broiler chow mixed with some rabbit chow pellets. Ducks were made anemic by repeated withdrawal of blood by

COMPARISON OF METHODS FOR MEASUREMENT OF AVIAN HEMOGLOBIN

By H. H. ROSTORFER

(*From the Department of Physiology, Indiana University, Bloomington*)

Discrepancies between the iron content, acid and alkaline hematin, and oxyhemoglobin, as determined by the manometric apparatus according to the method of Van Slyke and Neill (1), have been reported for avian blood by various investigators. Klein *et al.* (2) reported that values obtained by spectrographic analysis were less variable than the results of the Van Slyke analysis. However, the equilibrated samples were allowed to stand for some convenient time before analysis. This may have been a source of error, since avian red blood cells utilize considerable oxygen. Schultze and Elvehjem (3) developed a modified Newcomer method for acid hematin determination for avian blood. They compared the acid hematin readings expressed as gm. of hemoglobin with total iron values obtained by direct analysis of the bloods of the same individuals; and the average difference between the calculated oxyhemoglobin iron and the iron found analytically was 12.7 per cent extra iron. Even so, they believed the acid hematin method to be reliable. The extra amount of iron was believed to be non-hemoglobin iron, since a considerable amount, 5.4 per cent, of non-hemoglobin iron had been reported to be present in chicken blood by Winegarden and Borsook (4).

Bankowski (5) compared several photometric methods for acid and alkaline hematin prepared from chicken blood and believed the acid hematin method, compared photometrically with a standard prepared from crystalline acid hematin, to be the most accurate. The lowest values were found with the Dare hemoglobinometer, with an average of 7.72 gm. per 100 ml. of blood. The highest values, 12.13 gm. per 100 ml. of blood, were obtained on the same individuals by the use of alkaline hematin, read photometrically.

Rostorfer and Rigdon (6, 7) attempted to establish a linear relationship for avian blood between the log I_0/I for acid hematin, prepared according to the method of Schultze and Elvehjem and read at 450 mμ on the Coleman spectrophotometer, and oxyhemoglobin from the Van Slyke analysis. It was found that blood from mature ducks formed a linear relationship that diverged from a similar expression for blood obtained from young ducks 3 to 15 days old. These authors investigated the Van Slyke manometric method for measurement of avian blood and found the variation be-

5. Gemill, C. L., and Hamman, L., Jr., *Bull. Johns Hopkins Hosp.*, **68**, 50 (1941).
6. Gemill, C. L., *Bull. Johns Hopkins Hosp.*, **68**, 329 (1941).
7. Stadie, W. C., *Yale J. Biol. and Med.*, **16**, 539 (1944).
8. Stadie, W. C., and Zapp, J. A., Jr., *J. Biol. Chem.*, **170**, 55 (1947).
9. Price, W. H., Cori, C. F., and Colowick, S. P., *J. Biol. Chem.*, **160**, 633 (1945).
10. Price, W. H., Slein, M. W., Colowick, S. P., and Cori, G. T., *Federation Proc.*, **5**, 150 (1946).
11. Colowick, S. P., Cori, G. T., and Slein, M. W., *J. Biol. Chem.*, **168**, 583 (1947).
12. Corkill, A. B., and Nelson, J. F., *Med. J. Australia*, **1**, 172 (1947).
13. Krahl, M. E., and Cori, C. F., *J. Biol. Chem.*, **170**, 607 (1947).
14. Krahl, M. E., and Park, C. R., *J. Biol. Chem.*, **174**, 939 (1948).
15. Broh-Kahn, R. H., and Mirsky, I. A., *Science*, **106**, 148 (1947).
16. Meyerhof, O., and Geliazkowa, N., *Arch. Biochem.*, **12**, 405 (1947).
17. Stadie, W. C., and Haugaard, N., *J. Biol. Chem.*, **177**, 311 (1949).
18. Fuhrman, F. A., and Field, J., *Am. J. Physiol.*, **139**, 193 (1943).
19. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
20. Krebs, H. A., *Z. physiol. Chem.*, **217**, 191 (1933).
21. Field, J., in Potter, V. R., Methods of medical research, Chicago, **1**, 289 (1948).
22. Hastings, A. B., *Harvey Lectures*, **36**, 91 (1940–41).
23. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
24. Sjogren, B. T., Nordenskjold, T., Holmgren, H., and Mollerström, J., *Arch. ges Physiol.*, **240**, 427 (1938).
25. Deane, H. W., Nesbett, F. B., and Hastings, A. B., *Proc. Soc. Exp. Biol. and Med*., **63**, 401 (1946).
26. Somogyi, M., *J. Biol. Chem.*, **90**, 725 (1931).
27. Folin, O., and Wu, H., *J. Biol. Chem.*, **41**, 367 (1920).
28. Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919).
29. Kramer, B., and Gittleman, L. F., *J. Am. Med. Assn.*, **81**, 1171 (1923)
30. Snedecor, G. W., Statistical methods, Ames, 4th edition, 214 (1946).
31. van Uytvanck, P., *Arch. internat. pharmacod. et thérap.*, **41**, 160 (1931).
32. Heymans, C., and Casier, H., *Compt. rend. Soc. biol.*, **111**, 1078 (1932).
33. Magne, H., Mayer, A., and Plantefol, L., *Ann. physiol. et physicochim. biol.*, **8**, 51 (1932).
34. Cahn, T., *Ann. physiol. et physicochim. biol.*, **9**, 393 (1933).
35. Cahn, T., and Houget, J., *Ann. physiol. et physicochim. biol.*, **9**, 427 (1933).
36. Ronzoni, E., and Ehrenfest, E., *J. Biol. Chem.*, **115**, 749 (1936).

this concentration DNP does not appear to affect hexokinase activity. Accordingly the augmentation of oxygen consumption of rat diaphragm produced by this concentration of DNP does not involve altered consumption of glucose supplied by the external medium.

Increased glycogen breakdown in the presence of low concentrations of nitrated phenols has been shown by several investigators (31–36). This observation can now be extended to rat diaphragm in which the concentration of DNP evoking maximum augmentation of oxygen consumption also produced marked glycogenolysis.

The values for glycogenolysis may be compared with the theoretical amount expected to account for the increment of oxygen uptake produced by DNP. It was found that the average amount of glycogen broken down by incubation with DNP was 0.157 mg. per 100 mg. of wet weight per 2 hours, expressed as glucose. The average increment of O_2 uptake caused by the concentration of DNP used was 0.90 μl. of O_2 per mg. of wet weight per hour. Assuming the reaction, $C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2$, goes to completion, this amount of O_2 would burn 0.243 mg. of glucose per 100 mg. of wet weight per 2 hours. It can be seen qualitatively that this figure and the figure for the amount of glycogen broken down are of the same order of magnitude. Since balance sheet studies were not done to compare oxygen consumption and glycogenolysis, no further refinement can be obtained from this data.

SUMMARY

1. Glucose uptake of rat diaphragm in Kreb's Ringer-phosphate solution was unaffected by 4.76×10^{-5} M DNP, which evoked maximum augmentation of oxygen uptake. Therefore, it may be said that the activity of hexokinase under the conditions of these experiments is not affected by this concentration of DNP and no analogy can be made with insulin as to the effect on this enzyme.

2. Glycogen breakdown in rat diaphragm is greatly accelerated, and almost all glycogen disappears when incubated with 4.76×10^{-5} M DNP. This is in contrast to the moderate synthesis of glycogen occurring in control experiments. The increase in oxygen can be approximately accounted for by the increased glycogen breakdown occurring with DNP.

BIBLIOGRAPHY

1. Peiss, C. N., and Field, J., *J. Biol. Chem.*, **175**, 49 (1948).
2. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
3. Perlmutter, M., and Greep, R. O., *J. Biol. Chem.*, **174**, 915 (1948).
4. Gemill, C. L., *Bull. Johns Hopkins Hosp.*, **66**, 232 (1940).

DNP. The corresponding standard deviations were 0.091 and 0.089 respectively. These results of each run, together with data on initial and final glycogen content and certain statistics, are shown in Table I.

The error in any one determination of glucose concentration was considerable because variation of 1 division on the spectrophotometer scale changed the measurement by 0.04 or 0.05 mg. or about 14 per cent of the final reading. Accordingly the data were subjected to an analysis of variance, by the methods described by Snedecor (30). The variance due to treatment with DNP, to interanimal difference, intraanimal difference, and interaction was evaluated.

It was found that the difference due to treatment with DNP was not significant. There was no significant intraanimal difference. Thus it appears that neither treatment with 4.76×10^{-5} M DNP nor cutting the diaphragm into pieces of different size introduced serious error (cf. also Krahl and Cori (13)). However, the interanimal difference gave an F value (Snedecor's variance ratio) of 5.667 or a probability of 0.1 per cent.

Our values for glucose utilization by excised rat diaphragm (see Table I) are of the same order as those in the literature (3, 13, 14).

In this series, it was found that a small glycogenesis usually occurred during incubation, the means of the control and postincubation values being 0.171 and 0.230 mg. of glycogen per 100 mg. of wet weight per 2 hours (Table I). When incubated with DNP, however, glycogen was almost completely broken down. Since the limits of accuracy of the method of analysis for glucose after glycogen hydrolysis were approximately 0.009 to 0.010 mg., all values below this were deleted from the data. This accounted for almost 20 per cent of the results; however, ignoring these values can only minimize the difference, which is nevertheless sufficiently manifest.

Glycogen is expressed as mg. per 100 mg. of wet weight per 2 hours incubation. The mean of the values for glycogen content after incubation without DNP is 0.230 ± 0.076, and for that with DNP is 0.0282 ± 0.0154 (the second figure in each case is the standard deviation). From the data a t value of 3.95 was obtained and a probability of 0.0002. Hence treatment with DNP resulted in increased glycogen breakdown.

The values in the literature for glycogen before and after incubation are in accord with these figures (3, 8).

Thus it is evident from inspection of Table I that DNP exerts little influence on glucose utilization in the normal rat diaphragm, but does markedly accelerate glycogenolysis in this tissue.

DISCUSSION

Statistical analysis of our data has shown that 4.76×10^{-5} M DNP does not modify the rate of glucose utilization by isolated rat diaphragm. In

The protein-free filtrates were then analyzed for glucose by the method of Folin and Wu (27, 28), with use of the Coleman universal spectrophotometer with a 540 mμ filter. Samples of the original medium both with and without DNP were analyzed concurrently, and the glucose utilization was then determined by difference. In the case of the final glucose analysis

TABLE I

Effect of 4.76 × 10⁻⁵ M DNP on Glucose Uptake and Glycogenesis by Diaphragms of Normal Rats

Each line gives the measurements on one rat. The glucose concentration is 100 mg. per cent. Vessels at 37.5°, and incubated 2 hours. Values expressed as mg. per 100 mg. of wet weight of diaphragm per 2 hours. Each figure is the mean of two to three measurements, and the standard deviation is calculated from the values of the individual measurements rather than the means reported here.

Rat No	Sex	Without DNP		With DNP		Initial glycogen
		Glucose used	Final glycogen	Glucose used	Final glycogen	
1	F.		0.257		0.0288	0.208
2	"		0.270		0.0258	0.185
3	"	0.376	0.222	0.303	0.0320	0.203
4	M.	0.371	0.264	0.273	0.0240	0.116
5	"	0.278	0.194	0.283	0.0234	0.054
6	F.	0.230	0.138	0.266	0.0182	0.075
7	M.	0.265	0.246	0.241	0.0200	0.085
8	F.	0.263	0.252	0.185	0.0481	0.505
9	M.	0.380	0.246	0.333		0.254
10	"	0.424	0.178	0.376		0.102
11	"	0.388	0.391	0.412	0.0660	0.299
12	F.		0.146		0.0162	0.073
13	"		0.185		0.0139	0.069
14	M.	0.319		0.369		
15	"	0.500		0.453		
Mean.		0.345	0.230	0.318	0.0282	0.171
S.D. .		±0.089	±0.076	±0.091	±0.0154	

Test for significance of glycogenolysis with DNP (pair comparison), $t = 3.95$, $P = 0.0002$.

following glycogen hydrolysis, the amounts of glucose were often so small that the modification of Kramer and Gittleman (29) was used.

Results

Glucose consumption is expressed in terms of mg. of glucose consumed per 100 mg. of wet weight of diaphragm for a 2 hour interval. On this basis it was found that the mean glucose consumption by diaphragm was 0.318 in the presence of 4.76 × 10⁻⁵ M DNP and 0.345 in controls without

test-tubes were thoroughly chilled in an ice bath. 1 ml. portions of the aqueous carbohydrate solutions were then added and the resultant solutions mixed by stirring with a glass rod, the tubes being immersed in the ice bath to avoid a rise in temperature. These solutions, which were uniformly 79 per cent in sulfuric acid, were placed in a bath kept at 25° for 2 hours or in a boiling water bath for 15 minutes. The spectra of these solutions were determined, at room temperature, after cooling in a water bath, with a Beckman model DU spectrophotometer equipped with
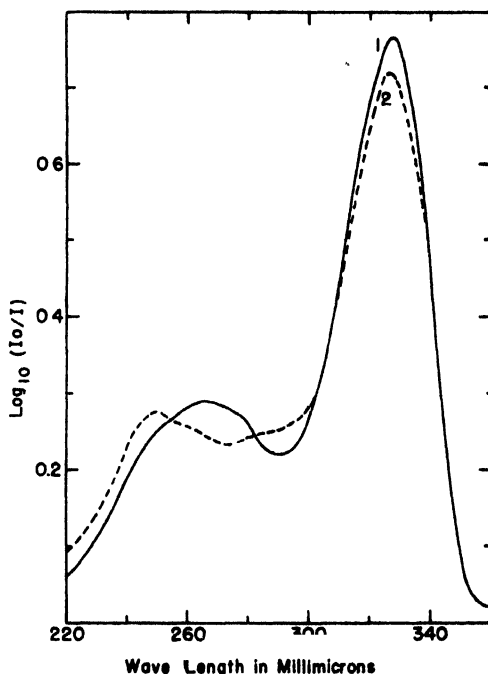
FIG. 2. Spectra of heated solutions of two 6-desoxyaldohexoses in 79 per cent sulfuric acid. Curve 1, 100 γ of rhamnose; Curve 2, 100 γ of fucose.

1 cm. quartz cells. The values reported are averages of two or more separate determinations, with a maximum deviation of ±5 per cent from the mean value. The curves were constructed from readings taken at 10 mμ intervals except in regions of maxima and minima, where the interval was reduced to 5 and 2.5 mμ.

The absorption curves for solutions of glucose, galactose, mannose, fructose, and N-acetylglucosamine in 79 per cent sulfuric acid, after these solutions had been heated at 100° for 15 minutes, have been presented earlier (6). The absorption curves for comparable solutions of xylose,

arabinose, ribose, lyxose, rhamnose, fucose, sorbose, glucosamine, glucurone, and galacturonic acid are given in Figs. 1 to 3, inclusive.

The salient spectral characteristics of 79 per cent sulfuric acid solutions of the carbohydrates investigated are given in Table I. Molecular extinction coefficients were calculated from the relation $\epsilon = 1/cd \log_{10} I_0/I$, where c = the final concentration in moles per liter of the solution and

Wave Length in Millimicrons
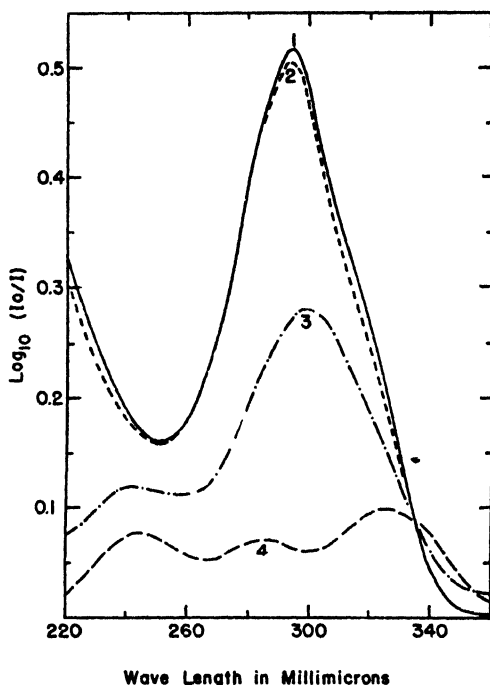
FIG. 3. Spectra of heated solutions of various sugars in 79 per cent sulfuric acid Curve 1, 100 γ of glucurone; Curve 2, 100 γ of galacturonic acid; Curve 3, 100 γ o- sorbose; Curve 4, 100 γ of glucosamine.

d = the cell thickness in cm. In Table I values of $\lambda_{max.}$ and $\epsilon_{max.}$ are given for solutions heated at 100° for 15 minutes; values of ϵ_{315} for solutions maintained at 100° for 15 minutes and at 25° for 2 hours, respectively, are recorded. Extinction coefficients were determined at 315 mμ, because practically all of the principal maxima lie in the region of 300 to 325 mμ and values obtained at 315 mμ appear to be generally useful for comparative purposes. In addition, fructose solutions maintained at 25°, which were of particular interest because of the unusual behavior of the keto-hexoses, absorb maximally at this wave-length.

The 2 hour period selected for observations at 25° was a compromise.

TABLE I

Spectral Characteristics of Solutions of Various Carbohydrates in 79 Per Cent Sulfuric Acid

Carbohydrate	15 min. at 100°		2 hrs. at 25°
	$\lambda_{max.}$	$\epsilon_{max.} \times 10^{-3}$	$\epsilon_{115\,m\mu} \times 10^{-3}$
	$m\mu$		
D-Xylose	309	14.3	13.6	2.7
	254	3.8		
D-Arabinose	307	8.3	7.7	0.46
	243	3.0		
D-Ribose	307	9.2	8.5	0.82
	242	3.5		
D-Lyxose	299.	6.6	5.7	1.26
	237	3.6		
L-Fucose	327	11.8	8.9	0.98
	250	4.6		
L-Rhamnose	327	12.6	9.5	1.00
	266	4.8		
D-Glucose	308	4.0	3.7	0.09
	250	1.6		
D-Mannose	305	2.3	2.2	0.11
	249	2.9		
D-Galactose	300	4.9*	3.6	0.13
	250	2.2		
D-Fructose	313	3.1	3.0	11.5
	255	1.4		
L-Sorbose	299	5.0	3.9	10.6
	243	2.2		
D-Glucurone	294	9.3	5.5	0.02
D-Galacturonic acid	294	9.8	5.6	0.17
N-Acetyl-D-glucosamine			0.31	0.0
D-Glucosamine hydrochloride	245	1.4	1.5	0.04
	287	1.25		
	325	1.75		
D-Mannitol			0.09	0.0
meso-Inositol			0.02	0.0
Adonitol			0.0	0.0
Sucrose			6.3	12.0
Maltose			6.9	0.17
Trehalose			6.9	0.03
Lactose			7.1	0.24
Melibiose			7.2	0.31
Raffinose			9.8	11.6
Melezitose			10.0	12.8
Starch			3.2†	0.02†
Glycogen			3.1†	0.06†
Inulin			2.6†	9.6†

* This value is about 0.8 higher than that calculated from a previous determination (6).

† Calculated by taking the anhydrohexose unit ($C_6H_{10}O_5$) as the molecular unit.

A period longer than 2 hours at 25° was not judged desirable, because solutions of the pentoses, notably xylose, began to absorb rather strongly and with a period of less than 2 hours the extinction coefficients of the solutions of sorbose and the fructose containing oligosaccharides were increasing too rapidly to permit the attainment of reproducible results. It was noted previously (6) that the extinction coefficients of the bands in the 300 to 325 mμ region decreased slowly when the solutions were maintained at 100° for prolonged periods. It appears that with a number of sugars $\epsilon_{max.}$ is attained in approximately 5 minutes heating at 100°, decreasing 10 to 15 per cent during the next few minutes of heating. 15 minutes at 100° appeared to be a reasonable compromise, considering convenience, reproducibility, and general applicability.

TABLE II

Behavior of Classes of Monosaccharides in 79 Per Cent Sulfuric Acid

Class	At 25°, 315 mμ		At 100°, 315 mμ	
	$\epsilon \times 10^{-3}$	$\text{Log}_{10} I_0/I^*$	$\epsilon \times 10^{-3}$	$\text{Log}_{10} I_0/I^*$
Aldopentoses and 6-Desoxyaldohexoses .	>0.4	>0.025	>5.0	>0.35
Aldohexuronic acids . .	<0.2	<0.010	>5.0	>0.25
Aldohexoses	<0.2	<0.010	<4.0	<0.25
Ketohexoses	>10.0	>0.550	<4.0	<0 25
Polyols.... .	<0.1	<0.005	<0.1	<0.005

* Of solutions containing 10 γ of anhydrous compound per ml. of acid solution

Results

From the data contained in Table I certain generalizations, summarized in Table II, may be made with respect to the behavior of non-nitrogenous monosaccharides in 79 per cent sulfuric acid. It is evident that the information contained in Table II will permit one to assign a given non-nitrogenous monosaccharide to a particular class and thus facilitate its ultimate characterization either by further spectrophotometric analysis, by means of the data contained in Table I, or by paper chromatography (10).

With binary mixtures of non-nitrogenous monosaccharides it follows from the data given in Table I that those mixtures which contain a keto-hexose or a 6-desoxyaldohexose as one component are amenable to analysis by the above spectrophotometric procedure. Binary mixtures of two members of the same class (Table II) in most cases cannot be analyzed satisfactorily, nor can binary mixtures containing only aldopentoses, aldo-hexoses, or aldohexuronic acids, though in these cases a value for $\log_{10}$ I_0/I greater than 0.25 for a solution containing 10 γ of solute per ml., heated at 100° and observed at 315 mμ, will be indicative of the presence of an appreciable amount of aldopentose or aldohexuronic acid.

A number of color reactions suitable for distinguishing between aldoses and ketoses and based upon treatment of the sugar with sulfuric or hydrochloric acid and a second reagent have been described (11). In view of the fact that adequate spectrophotometers are now generally available, it would appear from the present investigation that direct spectrophotometric examination of the acid solution without the addition of a second reagent is the more satisfactory procedure, especially for quantitative observations.

The applicability of the direct spectrophotometric method to oligosaccharides is illustrated by data contained in Table I. It will be noted that in general there is good agreement between the extinction coefficients

TABLE III

Analysis of Mixtures of Galactose and Fucose

Mixture	Amount		Moles galactose / Moles fucose	
	Added	Found*	Added	Found
	γ	γ		
Galactose	54.9	58	1.00	1.08
Fucose	50.0	49		
Galactose ..	82.4	84.5	3.00	3.21
Fucose .	25.0	24		
Galactose .	27.4	27	0.33	0.32
Fucose	75.0	77		
Galactose ..	54.9	57	2.00	2.16
Fucose	25.0	24		
N-Acetylglucosamine†	33.7			

* Average of a duplicate analysis.

† The absorption at 300 and 327 mμ was corrected for the slight amount due to the N-acetylglucosamine added.

of solutions of the oligosaccharides and those expected on the basis of the component monosaccharides. The data clearly show that maltose, trehalose, lactose, and melibiose contain 2 aldohexose residues; sucrose, an aldohexose and a ketohexose residue; and raffinose and melezitose, 1 ketohexose, and 2 aldohexose residues.

Whereas the ketohexoses are distinguishable from the other classes of sugars listed in Table II because of the large extinction coefficients developed in 79 per cent sulfuric acid solutions at 25°, the 6-desoxyaldohexoses are distinguishable from the other classes because of the position of the principal maxima developed in 79 per cent sulfuric acid solutions heated at 100°. It will be noted that the principal maxima of heated solutions of fucose and rhamnose are located at 327 mμ; *i.e.*, at a longer wave-length than the maxima of heated solutions of the other types of

monosaccharides. Fortunately the extinction coefficients of these maxima at 327 mμ are quite large, making it possible to resolve the spectra of solutions containing 6-desoxyaldohexose and other types of sugar into a "6-desoxyaldohexose peak" and a second peak characteristic of the other type of monosaccharide present in the mixture.

Fucose and rhamnose are known to occur in a number of immunologically active polysaccharides (12), and in studies in these laboratories on preparations of blood group A-specific substance from hog gastric mucosa it has been found that the amount of 6-desoxyaldohexose present in these preparations can be determined from the spectra of their solutions in 79 per cent sulfuric acid after the latter have been heated at 100° for 15 minutes. The values so obtained for the fucose and galactose content of preparations of blood group-A specific substance were in good agreement with values noted by other investigators.[1] Several representative analyses of fucose-galactose and fucose-galactose-N-acetylglucosamine mixtures are given in Table III. These values were calculated from extinction values found at 300 mμ (galactose) and 327 mμ (fucose) for solutions of the mixtures and of the pure monosaccharides.

The direct spectrophotometric determination of carbohydrates by the above procedure should be used with caution in the investigation of complex natural products until interferences arising from non-carbohydrate components can be evaluated. Although it has been found that the method is applicable for the estimation of carbohydrates in the presence of significant quantities of representative simple proteins,[1] no information is available in regard to possible interference by other classes of organic compounds.

SUMMARY

The spectral characteristics of solutions of eighteen representative monosaccharides in 79 per cent sulfuric acid have been determined for solutions maintained for 2 hours at 25° and 15 minutes at 100°. The use of these data for the identification of ketohexoses and 6-desoxyaldohexoses has been pointed out, as has their application to the quantitative determination of certain monosaccharides when present singly or in admixture with other monosaccharides, either as the monosaccharides or as components of oligosaccharides and polysaccharides.

BIBLIOGRAPHY

1. Bednarczyk, W., and Marchlewski, L., *Biochem. Z.*, **300**, 42 (1938).
2. Bosch, W. C., and Anderson, E. W., *Phys. Rev.*, **61**, 726 (1942).
3. Dische, Z., *Biochem. Z.*, **189**, 77 (1927).

[1] Unpublished studies of the authors.

4. Dische, Z., *Mikrochemie*, **8**, 4 (1930).
5. Sörensen, M., and Haugaard, G., *Biochem. Z.*, **260**, 247 (1933); *Compt.-rend. trav. Lab. Carlsberg*, **19**, No. 12 (1933).
6. Holzman, G., MacAllister, R. V., and Niemann, C., *J. Biol. Chem.*, **171**, 27 (1947).
7. Dische, Z., and Shettles, L. B., *J. Biol. Chem.*, **175**, 595 (1948).
8. Bandow, F., *Biochem. Z.*, **294**, 124 (1937).
9. Hammett, L. P., Physical organic chemistry, New York, 266 (1940).
10. Partridge, S. M., *Biochem. J.*, **42**, 238 (1948).
11. Tollens-Elsner, Kurzes Handbuch der Kohlenhydrate, Leipzig, 4th edition, 89–91 (1935).
12. Stacey, M., *Advances in Carbohydrate Chem.*, **2**, 164 (1946).

ESTERASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES*

BY R. J. ROSSITER AND ESTHER WONG

(From the Department of Biochemistry, University of Western Ontario, London, Canada)

Recently Cram and Rossiter (1) reported on the phosphomonoesterases of rabbit polymorphonuclear leucocytes. These cells also possess an enzyme capable of hydrolyzing the simple methyl esters and triglycerides of the low molecular weight fatty acids.

As early as 1909 Bergel (2) claimed that lymphocytes from tuberculous pus, spleen, or lymph glands contained an active lipase, an observation confirmed by Fiessinger and Marie (3). Nees (4) stated that polymorphonuclear leucocytes from pus also contained a lipase, but Bergel (5) claimed that the enzyme was in contaminating microorganisms rather than in the polymorphonuclear cells themselves. Morris and Boggs (6) showed that leucocytes from the blood of patients with both myeloid and lymphatic leucemia hydrolyzed ethyl butyrate. Subsequently Fleischmann (7) showed that rabbit polymorphonuclear leucocytes contained a tributyrin-splitting enzyme, while Barnes (8) reported that the enzyme was present in cat and rabbit lymphocytes as well as in rabbit polymorphonuclear leucocytes. All these workers referred to the enzyme as a *lipase*. The substrate specificity, kinetics, and Michaelis constant indicate that it should be called an *esterase* rather than by the more general and less accurate term *lipase*

A preliminary account of this work has already appeared (Rossiter and Wong (9)).

Methods

Obtaining Cells—Cells were obtained from the peritoneal cavity of the rabbit by the method of de Haan (10), the details of which have already been described (1). The preparations, which contained approximately 95 per cent polymorphonuclear cells, retained their activity for at least a week when stored in the refrigerator.

A measured volume of the peritoneal exudate was centrifuged for 5 minutes at 2000 R.P.M. and the cells washed three times with isotonic saline. They were then suspended in sufficient of the isotonic saline to

make the final count 30,000 cells per c.mm. when the substrate was tributyrin and 60,000 cells per c.mm. when it was methyl butyrate. Cell-free preparations, suitable for studying enzyme kinetics, were prepared by suspending the cells in 0.01 per cent alkyl sulfate[1] or 1.0 per cent saponin in isotonic saline. Rossiter (11) has shown that such surface-active substances liberate the enzyme from the cells into the surrounding fluid. If the cell débris is then removed by centrifuging, a clear enzyme solution can be obtained. For the determination of the enzyme in suspensions from different rabbits there was no obvious advantage in using cell-free preparations because the esterase activity, unlike phosphomonoesterase activity (Cram and Rossiter (1)), was no greater when surface-active substances were added to the cell suspensions.

Determination of Esterase Activity—The activity of the enzyme was determined by the method of Rona and Lasnitzki (12) in which the carbon dioxide, displaced from a bicarbonate buffer by the hydrogen ion liberated during the hydrolysis of the substrate, is measured in a standard Warburg manometer. To the main chamber of the manometer vessel were added 3.8 ml. of 0.025 M sodium bicarbonate and 1 ml. of enzyme preparation. To the side arm was added 0.2 ml. of either 0.25 M tributyrin or 1.0 M methyl butyrate emulsified in 5 per cent gum acacia in the bicarbonate buffer. The vessels were gassed with a mixture of 5 per cent carbon dioxide in nitrogen and placed in a water bath at 38°. After an equilibration period of 10 minutes the experiment was started by tipping the substrate from the side arm into the main chamber.

Recording of Results—Usually the enzyme activity was recorded as the quantity of carbon dioxide (in microliters) liberated by 1 ml. of enzyme solution in 1 hour. In the section on the quantitative determination of the enzyme in the leucocytes, the results are given as microliters of carbon dioxide per microgram of acid-insoluble phosphorus per hour. In order to bring these results into line with other estimates of the concentration of enzymes in white blood cells, they were also converted into microliters of carbon dioxide per 10^6 cells per hour, by the principle that 10^7 cells contain 6.8 γ of acid-insoluble phosphorus, previously established for the rabbit polymorphonuclear leucocyte (1).

Results

Quantitative Determination of Esterase in Rabbit Polymorphonuclear Leucocytes

Table I shows the esterase activity of suspensions of rabbit polymorphonuclear leucocytes when 0.01 M tributyrin was the substrate. Some idea

of the concentration of the enzyme in the cells can be obtained by comparing the activity of the esterase with that of the more familiar respiratory and glycolytic enzymes. The figure of 12.35 ± 1.50 μl. of carbon dioxide per microgram of acid-insoluble phosphorus per hour can be com-

TABLE I

Esterase Activity of Washed Rabbit Polymorphonuclear Leucocytes

Substrate, tributyrin 0.01 M; incubation time, 1 hour; temperature, 38°.

Per microgram acid-insoluble P	Per 10⁴ cells
μl. CO_2 per hr.	μl. CO_2 per hr.
12.5	8.5
11.0	7.4
10.9	7.3
15.4	10.3
14.0	9.5
13.3	9.1
11.9	8.1
12.8	8.8
11.4	7.8
10.3	7.1
Mean . 12.35	8.39
S.D... ±1.50	±0.99

TABLE II

Esterase Activity of Washed Rabbit Polymorphonuclear Leucocytes

Substrate, methyl butyrate 0.04 M; incubation time, 1 hour; temperature, 38°

Per microgram acid-insoluble P	Per 10⁴ cells
μl. CO_2 per hr.	μl. CO_2 per hr.
5.6	3.8
6.6	4.6
3.9	2.7
8.8	6.0
3.8	2.6
Mean .. 5.74	3.94
S.D.... . ±1.86	±1.27

pared with an oxygen uptake of 0.47 μl. of oxygen per microgram of acid-insoluble phosphorus per hour and an anaerobic glycolysis of 2.23 μl. of carbon dioxide per microgram of acid-insoluble phosphorus per hour for a similar suspension.

Table II, giving similar figures when 0.04 M methyl butyrate was the

substrate, shows that the activity with methyl butyrate was less than with tributyrin. Both substrates were present in optimal concentrations.

Concentration of Enzyme—Fig. 1 shows that, under the conditions of study, the rate of carbon dioxide liberation was proportional to the concentration of the enzyme.

Time Course of Reaction—With tributyrin as substrate the course of the reaction was linear with time, provided the substrate concentration was greater than 0.005 M (Fig. 2). For lower concentrations of tributyrin the activity fell off after the first 20 minutes, reaching zero when 60 to 80 per cent of the substrate had been hydrolyzed. Other workers have reported that the hydrolysis of tributyrin by either liver esterase (12–14) or pan-
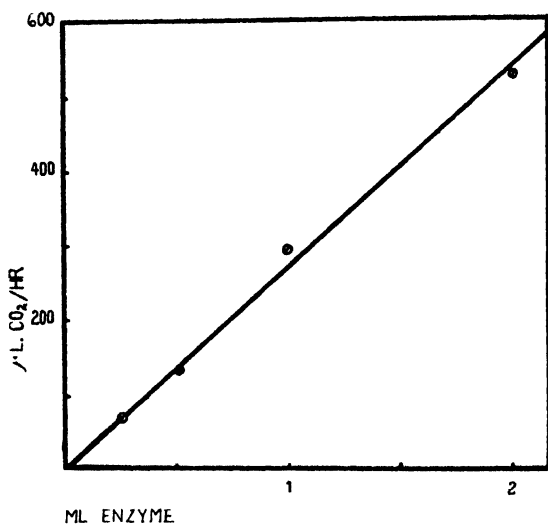
FIG. 1. The relation between enzyme activity and concentration of rabbit polymorphonuclear leucocyte esterase Substrate, 0.01 M tributyrin; temperature, 38°

creatic lipase (13–15) was linear with time. For pancreatic lipase the velocity of the reaction fell off when only a small percentage of the substrate had been hydrolyzed, but this was not so for liver esterase (13, 15). In this respect the enzyme of the rabbit polymorphonuclear leucocyte resembles liver esterase rather than pancreatic lipase.

When the substrate was methyl butyrate, however, the course of the reaction with time was different (Fig. 3). For methyl butyrate concentrations of 0.04 M or higher the reaction was linear with time for periods up to an hour. When the substrate concentration was lower than 0.04 M, the activity fell off rapidly and usually became zero when only 4 to 8 per cent of the substrate had been hydrolyzed. Experiments with 0.005 M
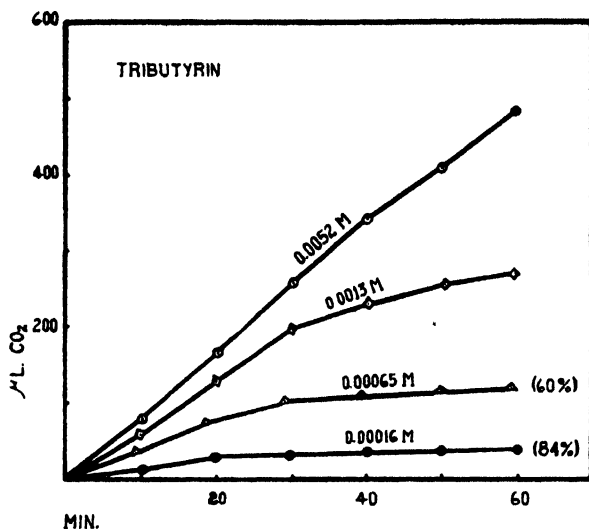
FIG. 2. Time course of reaction for rabbit polymorphonuclear leucocyte esterase. Substrate, tributyrin; temperature, 38°.
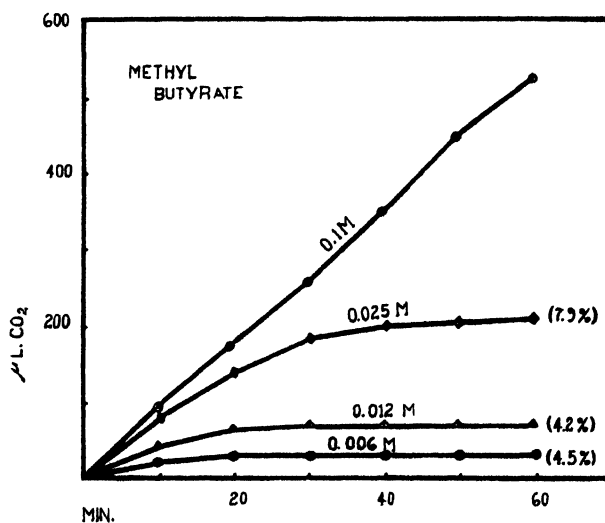
FIG. 3. Time course of reaction for rabbit polymorphonuclear leucocyte esterase. Substrate, methyl butyrate; temperature, 38°.

methyl alcohol or 0.005 M sodium butyrate, *i.e.* concentrations well in excess of those formed during the reaction, showed that the products of the reaction could not be the cause of this inhibition. It was possible that

either the enzyme or the substrate was inactivated during the course of the reaction. In the experiment reported in Fig. 4 either fresh substrate or fresh enzyme was added to the reaction mixture at the time when the activity had fallen almost to zero. The addition of fresh substrate restored the activity to normal (Curve I), whereas the addition of fresh enzyme had no such effect (Curve II). Neither the enzyme nor the substrate was
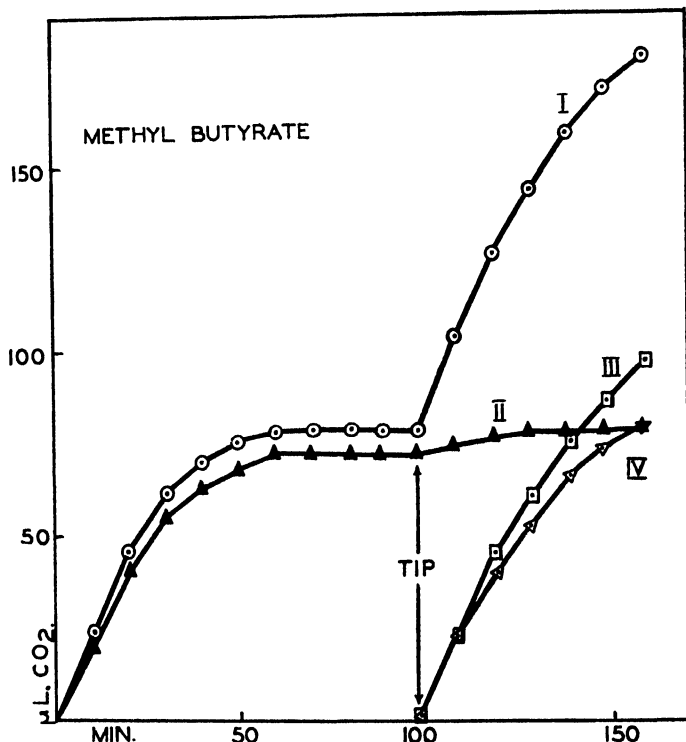
FIG. 4. Effect of adding fresh substrate or enzyme to rabbit polymorphonuclear leucocyte esterase. Substrate, methyl butyrate 0.005 M; temperature, 38°. Curve I, fresh substrate tipped into reaction mixture at 100 minutes; Curve II, fresh enzyme tipped into reaction mixture at 100 minutes; Curve III, substrate tipped into reaction mixture to which no substrate had previously been added; Curve IV, enzyme tipped into reaction mixture to which no enzyme had previously been added.

inactivated when incubated alone, for when substrate was added to enzyme that had been incubated with no substrate (Curve III), or when enzyme was added to substrate that had been incubated with no enzyme (Curve IV), normal activity resulted. This would indicate, as has been postulated by Sobotka and Glick (13) for pancreatic lipase, that the substrate combined with inert material in the enzyme preparation, or perhaps

with inactive groups on the enzyme itself. When more substrate was added, the reaction was able to proceed normally.

Substrate Concentration—Fig. 5 shows the effect of substrate concentration on the enzyme when either methyl butyrate or tributyrin was the substrate. Excess methyl butyrate inhibited the enzyme, but excess tributyrin did not. From data such as are presented in Fig. 5 the Michaelis constants, K_s, for each of the two substrates can be calculated by the method of Lineweaver and Burk (16). Fig. 6 shows that the plot of $1/V$ against $1/S$ was a straight line.

Although other workers have calculated K_s values for enzymes hydrolyzing esters of butyric acid (13, 17, 18), the results have to be interpreted with caution because of the insolubility of the substrates. Sobotka and
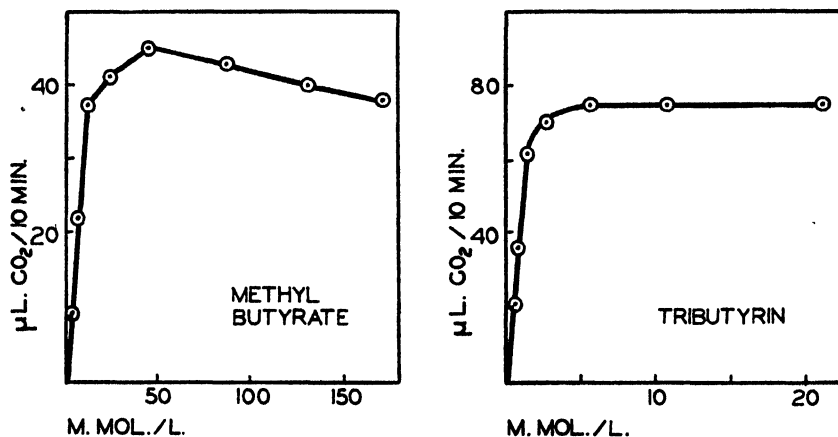
FIG. 5. The effect of substrate concentration on the activity of rabbit polymorphonuclear leucocyte esterase. Substrate, methyl butyrate or tributyrin; temperature, 38°.

Glick (13) have shown that, in the presence of the enzyme, the solubility of the substrate is greater, but during the course of the reaction it is impossible to tell how much of the substrate is in true solution. Also tributyrin has three ester linkages, each of which can presumably form a Michaelis-Menten enzyme-substrate complex, and the same is true of the ester linkages of the products of incomplete hydrolysis of tributyrin. We prefer, therefore, to speak of "apparent K_s," rather than K_s. We believe our estimate of the apparent K_s for tributyrin to be reasonably accurate. This is not so for methyl butyrate, for, with lower concentrations of the substrate, the observation period had to be extremely short if true initial velocities were to be measured and, for the higher substrate concentrations there was the complicating factor that excess of the substrate was in-

hibitory. With two different enzyme preparations the apparent K_s for tributyrin was 0.00039 and 0.00037 and that for methyl butyrate was 0.0072 and 0.0069.

When the logarithm of the substrate concentration was plotted against the activity of the enzyme, with methyl butyrate as substrate, a bell-shaped curve resulted, which Haldane (19) considers typical of inhibition by excess of the substrate (Fig. 7). He suggests that inhibition of an enzyme by excess substrate is due to 1 or more molecules of the substrate combining with the dissociable enzyme-substrate complex to form a further addition
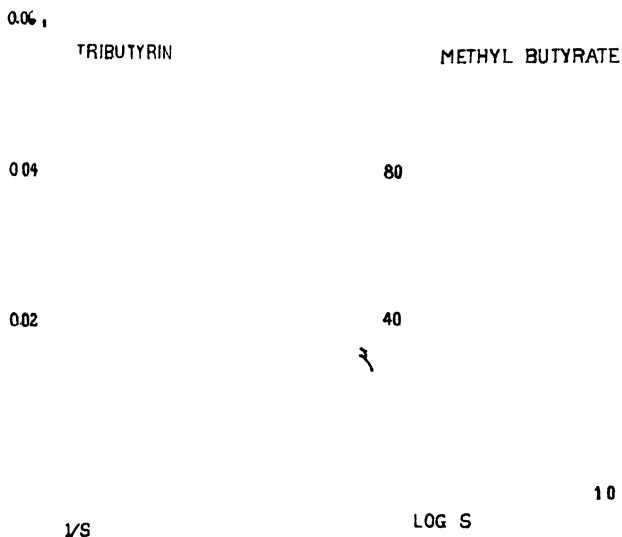
FIG. 6. Plot of $1/V$ against $1/S$ for rabbit polymorphonuclear leucocyte esterase. V is the initial velocity in microliters of CO_2 per 10 minutes and S is the substrate concentration in gm. molecules per liter. Substrate, tributyrin; temperature, 38°.

FIG. 7. Plot of activity against log S for rabbit polymorphonuclear leucocyte esterase. S is the substrate concentration in gm. molecules per liter. Substrate, methyl butyrate; temperature, 38°.

compound, which does not break down to give the products of the reaction. Thus we have

$$E + S \; : ES \text{ (active)}$$
$$ES + S \; : \; ES_2 \text{ (inactive)}$$
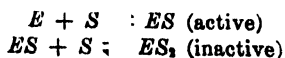
where ES dissociates into the products of the reaction and ES_2 is inactive and does not dissociate. The constant of the first reaction is K_s and that for the second can be represented by K_2. A mathematical development of

the theory gives

$$\frac{S}{V} = \frac{K_s}{V_M} + \frac{1}{V_M}\left(S + \frac{S}{K_2}\right)$$

where S is the concentration of the substrate, V_M is the maximum velocity, and V the observed initial velocity of hydrolysis (Lineweaver and Burk (16), Case III). In one experiment, in which higher substrate concentrations than those of the experiment reported in Fig. 5 were used, the apparent K_2 for the rabbit polymorphonuclear leucocyte enzyme was evaluated. The mean value was 0.68, but the inaccuracies in the determination of the apparent K_s and those inherent in the method of evaluating the apparent K_2 were such that, in this instance, it was impossible to test the validity of the Haldane theory. Other workers have also reported that an excess of the esters of the low molecular weight fatty acids inhibited liver esterase (18, 20).

Mixed Substrates—The enzyme is able to hydrolyze either tributyrin or methyl butyrate. Table III shows that, when these two substrates were added to the reaction mixture together, the activity was that of the highest, tributyrin. There was no indication of an additive effect. This would suggest that, despite many differences, such as the percentage of substrate hydrolyzed, the affinity of the substrate for the enzyme, and inhibition by excess substrate, each of the two substrates is hydrolyzed by the same enzyme.

Other Substrates—Table IV shows the esterase activity towards the methyl esters of a homologous series of fatty acids. With methyl formate there was considerable spontaneous hydrolysis, allowance for which rendered the figures for this ester less accurate. Generally the activity was greater as the length of the carbon chain increased up to methyl caprylate, the ester of the C_8 acid, which was the highest tested. Tween 20,[2] an ester of the C_{12} lauric acid used by Archibald (21) as a water-soluble substrate for lipase determinations, was only slightly hydrolyzed, while triolein was not hydrolyzed at all.

Choline Esters—Table V shows that suspensions of rabbit polymorphonuclear leucocytes, even when concentrated to 150,000 cells per c.mm., did not hydrolyze 0.003 M acetylcholine, but that a suspension of mouse brain under the same conditions was highly active. Since it was possible that the cells might not be freely permeable to acetylcholine, the experiment was repeated with a cell-free preparation of the enzyme (Table VI). Methyl butyrate and tributyrin were hydrolyzed readily; yet neither

[2] Obtained from the Atlas Powder Company, Wilmington, Delaware; described as "a mixture of polyoxyalkylene derivatives of an ester of 1 mole of fatty acid per mole of sorbitan."

TABLE III

Effect of Mixed Substrates on Esterase Activity of Cell-Free Enzyme Preparation (0.01 Per Cent Alkyl Sulfate) from Rabbit Polymorphonuclear Leucocytes

Incubation time, 1 hour; temperature, 38°.

Substrate	Preparation A	Preparation B
	$\mu l.\ CO_2\ per\ ml.$ $per\ hr.$	$\mu l.\ CO_2\ per\ ml.$ $per\ hr.$
Tributyrin, 0.01 M	105	202
Methyl butyrate, 0.04 M .	36	85
Tributyrin, 0.01 M + methyl butyrate, 0.04 M ..	91	206

TABLE IV

Esterase Activity of Cell-Free Enzyme Preparation (0.01 Per Cent Alkyl Sulfate) from Rabbit Polymorphonuclear Leucocytes

Substrate concentration 0.04 M; incubation time, 1 hour; temperature, 38°.

Substrate	Preparation A	Preparation B
	$\mu l.\ CO_2\ per\ ml.\ per\ hr.$	$\mu l.\ CO_2\ per\ ml.\ per\ hr.$
Methyl formate .	17	18
" acetate. . ..	12	8
" propionate	54	62
" butyrate . ..	120	161
" caprylate .	153	177
" malonate	288	396

TABLE V

Cholinesterase Activity of Suspensions of Washed Rabbit Polymorphonuclear Leucocytes

Substrate, acetylcholine 0.003 M; incubation time, 1 hour; temperature, 38°.

Preparation	Concentration of enzyme preparation	Cholinesterase activity
		$\mu l.\ CO_2\ per\ ml.$ $per\ hr.$
Rabbit polymorphonuclear leucocytes.	50,000 cells per c.mm.	0
	150,000 " " "	2
Mouse brain suspension	Diluted 1:25	357

TABLE VI

Esterase Activity of Cell-Free Enzyme Preparation (1 Per Cent Saponin) from Rabbit Polymorphonuclear Leucocytes

Incubation time, 1 hour; temperature, 38°.

Substrate	Substrate concentration	Esterase activity
	M	$\mu l.\ CO_2\ per\ ml.\ per\ hr.$
Acetylcholine	0.003	0
Acetyl-β-methylcholine	0.03	0
Benzoylcholine...	0.006	0
Methyl butyrate..	0.04	540
Tributyrin........	0.01	798

acetylcholine, acetyl-β-methylcholine, nor benzoylcholine[*] was hydrolyzed at all. It is possible that the saponin used to make the cell-free enzyme preparation might have inhibited a cholinesterase that was present, but since 1 per cent saponin had only a negligible inhibitory effect on the cholinesterases of human serum or red blood cells, this would appear to be unlikely. Thus the rabbit polymorphonuclear leucocytes are devoid of true cholinesterase, pseudocholinesterase (22, 23), or specific benzoylcholinesterase (24, 25). The cells also contain no atropine esterase, for other experiments showed that cell suspensions, even from rabbits whose sera were active (26, 27), were unable to hydrolyze atropine sulfate.

DISCUSSION

As early as 1906 Loevenhart (28) distinguished between pancreatic lipase and liver esterase, but, despite this, the terms lipase and esterase have since been used interchangeably by a great many workers. Although it is realized that an enzyme cannot be characterized for certain until it has been isolated in a pure state, a difficult procedure for leucocyte enzymes when so little material is available, we feel that present evidence is sufficient to justify calling the enzyme of rabbit polymorphonuclear leucocyte an esterase rather than a lipase.

Both the time course of the reaction with tributyrin and the effect of substrate concentration are typical of an esterase and not of a lipase. The apparent K_s of 0.00039 and 0.00037 for tributyrin agrees with the finding of Sobotka and Glick (13) that the K_s of pig liver esterase was 0.00035 for tributyrin. The apparent K_s of 0.0072 and 0.0069 for methyl butyrate is in keeping with the findings of Bamann and Schmeller (17) who reported values ranging from 0.001 to 0.03 for the K_s for methyl butyrate of the liver esterases from a number of different species. For pancreatic lipase, on the other hand, the values of K_s were higher (13), 0.0006 for tributyrin and >0.09 for methyl butyrate. Thus the pattern of apparent K_s of the enzyme of rabbit polymorphonuclear leucocyte resembles that of an esterase rather than that of a lipase. The inhibition of the enzyme by excess substrate is also a typical esterase phenomenon (18, 20).

The figures for substrate specificity are a little more equivocal. Most workers agree that liver esterase hydrolyzes the simple esters and triglycerides of the low molecular weight fatty acids and is inactive towards the higher triglycerides, while pancreatic lipase, as well as hydrolysing the esters and triglycerides of the low molecular weight fatty acids, also hydrolyzes the higher triglycerides (13, 28–30). Whether the hydrolysis of the simple esters is brought about by the lipase itself or by an esterase contaminating most lipase preparations is unknown. That the polymorpho-

[*] Kindly provided by Hoffmann-La Roche, Inc.

nuclear leucocyte enzyme hydrolyzed simple esters and not the higher triglycerides, such as triolein, is in favor of its being an esterase, but the pattern of activity for the lower esters resembled that reported for the pancreas enzyme (31-33).

The effect of inhibitors on the enzyme of rabbit polymorphonuclear leucocyte, which will form the subject of a further report, is characteristic of an esterase rather than a lipase.

Gomori (34, 35) described a histochemical test for lipase, in which he used as substrate either one of the Tweens or Product 81, stated to be a "stearic ester of comparatively short chained polyglycols." Although a number of tissues gave a positive reaction, white blood cells were uniformly negative. This is in keeping with our finding that the rabbit polymorphonuclear leucocyte did not hydrolyze the higher triglycerides and was only slightly active toward Tween 20.

Our inability to demonstrate an enzyme in the rabbit polymorphonuclear leucocyte that could hydrolyze choline esters is surprising, for most tissues possess some type of cholinesterase (36). However, the observation agrees with the finding of Ginsberg, Kohn, and Necheles (37) that there was no cholinesterase in the polymorphonuclear leucocytes of pus from an empyema. Also Colling and Rossiter[4] found no significant correlation between the total, true, or pseudocholinesterase activity of pathological cerebrospinal fluids and the concentration of white blood cells, while Brauer and Hardenbergh (38) found little cholinesterase activity in dog lymphocytes, and Youngstrom (39) found no cholinesterase in a mass of macrophages from a brain tumor.

Esterases occur in a variety of tissues (33, 40, 41), including plasma, in which they have been studied in great detail (42–47). Besides small quantities of lipase (30, 46), there are at least two enzymes in the plasma capable of hydrolyzing tributyrin and the esters of the low molecular weight fatty acids: pseudocholinesterase, which can also hydrolyze either acetylcholine or benzoylcholine, and ali-esterase or common esterase, which is inactive toward the choline esters (22, 23, 45). The enzyme of the polymorphonuclear leucocytes can be considered to be an ali-esterase or common esterase.

SUMMARY

1. Rabbit polymorphonuclear leucocytes contain an enzyme that is capable of hydrolyzing tributyrin or methyl butyrate.

2. The rate of hydrolysis of tributyrin was more than twice that of methyl butyrate.

3. Under the conditions of study the rate of hydrolysis was proportional to the concentration of the enzyme.

[4] Colling, K. G., and Rossiter, R. J., to be published.

4. Provided the substrate concentration was great enough, the rate of hydrolysis was linear with time for periods up to 1 hour. With methyl butyrate the activity of the enzyme fell off when only 4 to 8 per cent of the substrate had been hydrolyzed.

5. The Michaelis constant, K_s, for both tributyrin and methyl butyrate was evaluated. Excess of methyl butyrate, but not of tributyrin, inhibited the enzyme.

6. The enzyme hydrolyzed the triglycerides and simple esters of the low molecular weight fatty acids but not the higher triglycerides, such as triolein. It was also unable to hydrolyze acetylcholine, acetyl-β-methylcholine, benzoylcholine, or atropine sulfate.

7. The time course of the reaction, the Michaelis constant, and the substrate specificity are all consistent with the enzyme being an esterase rather than a lipase. It can be considered as an ali-esterase or common esterase.

BIBLIOGRAPHY

1. Cram, D. M., and Rossiter, R. J., *Biochem. J.*, **43**, p. xxi (1948); *Canad. J. Res.*, in press.
2. Bergel, S., *Münch. med. Wochschr.*, **56**, 64 (1909).
3. Fiessinger, N., and Marie, P., *Compt. rend. Soc. biol.*, **67**, 107 (1909).
4 Nees, F., *Biochem. Z.*, **124**, 156 (1921).
5. Bergel, S., *Biochem. Z.*, **130**, 533 (1922).
6. Morris, R. S., and Boggs, T. R., *Arch. Int. Med.*, **8**, 806 (1912)
7. Fleischmann, W., *Biochem. Z.*, **200**, 25 (1928).
8. Barnes, J. M., *Brit. J. Exp. Path.*, **21**, 264 (1940)
9. Rossiter, R. J., and Wong, E., *J. Physiol.*, in press.
10. de Haan, J., *Arch. néerl. physiol.*, **2**, 674 (1918).
11. Rossiter, R. J., *J. Physiol.*, in press.
12. Rona, P., and Lasnitzki, A., *Biochem. Z.*, **152**, 504 (1924).
13. Sobotka, H., and Glick, D., *J. Biol. Chem.*, **105**, 199 (1934).
14. Rona, P, and Ammon, R., *Biochem. Z.*, **181**, 49 (1927).
15. Murray, D. R. P., *Biochem. J.*, **23**, 292 (1929).
16. Lineweaver, N., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).
17. Bamann, E., and Schmeller, M., *Z. physiol. Chem.*, **183**, 149 (1929).
18. Murray, D. R. P., *Biochem. J.*, **24**, 1890 (1930).
19. Haldane, J. B. S., Enzymes, Monographs on biochemistry, London (1930).
20. Schwab, G.-M., Bamann, E., and Laeverenz, P., *Z. physiol. Chem.*, **215**, 121 (1933).
21. Archibald, R. M., *J. Biol. Chem.*, **165**, 443 (1946).
22. Mendel, B., and Rudney, H., *Biochem. J.*, **37**, 59 (1943).
23. Mendel, B., Mundell, D. B., and Rudney, H., *Biochem. J.*, **37**, 473 (1943).
24. Sawyer, C. H., *Science*, **101**, 385 (1945).
25. Gunter, J. M., *Nature*, **157**, 369 (1946).
26. Bernheim, F., and Bernheim, M. L. C., *J. Pharmacol. and Exp. Therap*, **64**, 209 (1938).
27. Glick, D., and Glaubach, S., *J. Gen. Physiol.*, **25**, 197 (1941).
28. Loevenhart, A. S., *J. Biol. Chem.*, **2**, 427 (1906).

29. Willstätter, R., and Memmen, F., *Z. physiol. Chem.*, **138,** 216 (1924).
30. Cherry, I. S., and Crandall, L. A , *Am. J. Physiol.*, **100,** 266 (1932).
31. Weinstein, S. S., and Wynne, A. M., *J. Biol. Chem.*, **112,** 649 (1936).
32. Balls, A. K., and Matlack, M. B., *J. Biol. Chem.*, **123,** 679 (1938).
33. Huggins, C., and Moulton, S. H., *J. Exp. Med.*, **88,** 169 (1948).
34. Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, **58,** 362 (1945).
35. Gomori, G., *Arch. Path.*, **41,** 121 (1946).
36. Sawyer, C. H., and Everett, J. W., *Am. J. Physiol.*, **148,** 675 (1947).
37. Ginsberg, R., Kohn, R., and Necheles, H., *Am. J. Digest. Dis. and Nutr.*, **4,** 154 (1937).
38. Brauer, R. W., and Hardenbergh, E., *Am. J. Physiol.*, **150,** 746 (1947).
39. Youngstrom, K. A., *J. Neurophysiol.*, **4,** 473 (1941).
40. Porter, A. E., *Biochem. J.*, **10,** 523 (1916).
41. Falk, K. G., Noyes, H. M., and Sugiura, K. U., *J. Biol. Chem.*, **59,** 183 (1924).
42. Hall, G. E., and Lucas, C. C., *J. Pharmacol. and Exp. Therap.*, **61,** 10 (1937).
43. Stedman, E., Stedman, E., and Easson, L. H., *Biochem. J.*, **26,** 2056 (1932).
44. Stedman, E., Stedman, E., and White, A. C., *Biochem. J.*, **27,** 1055 (1933).
45. Richter, D., and Croft, P. G., *Biochem. J.*, **36,** 746 (1942).
46. Lagerlöf, H., *Acta med. Scand.*, **120,** 407 (1945).
47. Huggins, C., and Lapides, J., *J. Biol. Chem.*, **170,** 467 (1947).

THE PRIMARY AND SECONDARY COMPOUNDS OF CATALASE AND METHYL OR ETHYL HYDROGEN PEROXIDE

IV. REACTIONS WITH HYDROGEN PEROXIDE*

BY BRITTON CHANCE†

(From the Biochemical Department of the Nobel Medical Institute, Stockholm, Sweden)

Stern (1) has found that as much as 0.13 N ethyl hydrogen peroxide is required to give 50 per cent inhibition of the destruction of hydrogen peroxide by catalase. This is difficult to understand in view of the high affinities of catalase for ethyl hydrogen peroxide in both the primary and secondary compounds ($\sim 2 \times 10^{-6}$ M) (2), in which all the catalase hematins are bound to ethyl hydrogen peroxide (3). Stern (1) also tested the inhibition of catalase activity by methyl hydrogen peroxide but found that methyl hydrogen peroxide disappeared in some unexplained fashion.

Since the activity of catalase is inhibited at a concentration of cyanide predicted by the spectrophotometric equilibrium constant (4) and both cyanide and peroxide attach to the iron atom of catalase (3), there must be some reaction which is responsible for the small inhibition by the alkyl hydrogen peroxides.

This paper describes a rapid reaction of hydrogen peroxide and catalase-bound alkyl hydrogen peroxide and presents some mechanisms for catalase activity.

Method

These reactions were all carried out in the rapid flow apparatus (5) and consist of mixing catalase, saturated with alkyl hydrogen peroxide, with hydrogen peroxide in the capillary of the rapid flow apparatus. If there is no reaction of catalase alkyl hydrogen peroxide with hydrogen peroxide, no effect will be observed except that caused by the dilution of the alkyl hydrogen peroxide, which is usually avoided by adding alkyl hydrogen peroxide to the hydrogen peroxide solution. The catalase-alkyl hydrogen peroxide complex must be stable between the first addition of alkyl hydrogen peroxide and the mixing with hydrogen peroxide in the rapid flow apparatus. These conditions were satisfactorily fulfilled with ethyl hydrogen peroxide (3) and are easy to fulfil with methyl hydrogen peroxide.

If a reaction of catalase alkyl hydrogen peroxide with hydrogen peroxide occurs, it is necessary to measure the extent of the reaction and to

---

* This is Paper 6 of a series on catalases and peroxidases.

† John Simon Guggenheim Memorial Fellow (1948). Present address, Johnson Research Foundation, University of Pennsylvania, Philadelphia.

identify any compounds formed in this reaction. It is therefore necessary to include a number of calibrations and controls: the optical densities of catalase, saturated catalase alkyl hydrogen peroxide, and saturated catalase hydrogen peroxide. Also the disappearance of hydrogen peroxide caused by catalase under the conditions of the experiment is indicated by the platinum microelectrode.[1]

Reaction of Catalase Ethyl Hydrogen Peroxide with Hydrogen Peroxide

In Fig. 1, A, B, and C represent calibrations preliminary to the final experiment, D. In A, the kinetics of formation and disappearance of catalase hydrogen peroxide are recorded spectrophotometrically at 405 mμ. As is shown by the middle tracing, the observation tube is initially filled with free catalase remaining from the previous experiment in which an excess of ethanol is present. On initiation of the flow, the catalase-hydrogen peroxide complex forms and remains at its saturation value until the flow stops. At this time, the peroxidatic reaction with the excess alcohol causes the complex to disappear in about 15 seconds. The lowest tracing represents the kinetics of hydrogen peroxide disappearance as recorded by the platinum microelectrode.[1] Catalase present in the observation tube from the previous experiment has completely decomposed the hydrogen peroxide used then, so that the initial hydrogen peroxide concentration is zero. On initiation of the flow, the breakdown of hydrogen peroxide by catalase is so rapid that even at the highest value of flow velocity (corresponding to a time after mixing of about 5 milliseconds) only about half of the initial hydrogen peroxide remains undecomposed, as is shown by the small downward deflection of the platinum microelectrode tracing. As the flow stops, the hydrogen peroxide concentration falls to zero, as shown by the rise of the platinum microelectrode tracing.

This record is of especial interest because it verifies the previous assumption (6) that the free hydrogen peroxide concentration falls to a very low value before the catalase-hydrogen peroxide complex begins to decompose.

In record B, Fig. 1, the reaction of catalase and ethyl hydrogen peroxide is shown. The observation tube is again initially filled with free catalase, but, since this reaction is relatively slow, the catalase-ethyl hydrogen peroxide complex does not form appreciably until after the flow stops. Then a large decrease of optical density is indicated by the downward deflection. Ethanol is omitted in this experiment to insure that the saturation value of the complex is recorded. In this and the following records, the amplifier sensitivity is decreased to half the value used in record A. Since ethyl hydrogen peroxide does not affect the platinum microelectrode, no deflection of that tracing is seen.

[1] Chance, unpublished data.

In record C, Fig. 1, catalase is mixed with both hydrogen peroxide and ethyl hydrogen peroxide. Initially the observation tube is filled with free catalase. On initiating the flow, the catalase-hydrogen peroxide complex forms immediately. The decrease of optical density is only about one-fourth[2] of that in record B because this complex consists of only 1 hydrogen peroxide molecule per catalase molecule (3). At the same time, the downward deflection of the platinum microelectrode record shows the presence of free hydrogen peroxide in the observation tube. As the flow stops, ethyl hydrogen peroxide combines with the free catalase hematins,
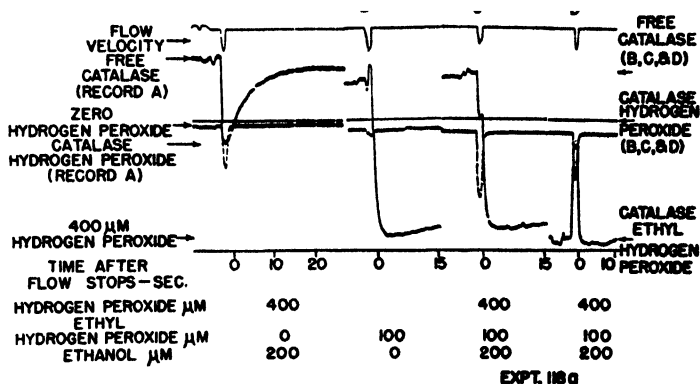
FIG. 1. Reactions of catalase ethyl hydrogen peroxide with hydrogen peroxide. In records A, B, and C, 0.76 μM of guinea pig liver catalase is mixed with the reactants listed beneath each record. In record D, catalase ethyl hydrogen peroxide is preformed and is then mixed with the reactants listed. The kinetics of the complexes are recorded spectrophotometrically at 405 mμ (the sensitivity in record A is twice that in B, C, and D). The kinetics of hydrogen peroxide disappearance are recorded by the platinum microelectrode as dashed tracings (portions of the original records were retouched for clarity). The microelectrode was polarized at +0.7 volt. pH 6.5; 0.01 M phosphate buffer.

as indicated by the further large decrease in optical density. This record shows negligible inhibition of catalase activity, because most of the hydrogen peroxide is decomposed before the ethyl hydrogen peroxide has time to combine with the catalase hematins.

In record D, Fig. 1, catalase is first mixed with ethyl hydrogen peroxide in a test-tube in the absence of ethanol, essentially duplicating the conditions of record B. Thereby catalase hematins are all bound to ethyl hydrogen peroxide, and the complex will be held at the saturation value

[2] This sample of guinea pig liver catalase contained very little bile pigment and hence nearly four intact hematins.

for several minutes. Catalase activity towards hydrogen peroxide would now be expected to be zero, since the catalase-hydrogen peroxide complex can only form by displacing ethyl hydrogen peroxide groups from catalase hematins. Since ethanol is omitted in this "premixing," the catalase-ethyl hydrogen peroxide complex is spontaneously decomposing very slowly, $k_3 = 0.02$ sec.$^{-1}$; a time of about 35 seconds would be required for catalase to regain half its normal activity. This mixture containing the catalase-ethyl hydrogen peroxide complex and excess ethyl hydrogen peroxide is then rapidly introduced into one of the syringes of the rapid flow apparatus and is mixed with hydrogen peroxide, as record D shows. The observation tube is initially filled with the catalase-ethyl hydrogen peroxide complex remaining from a previous identical experiment carried out a few seconds earlier. On initiating the flow and mixing the catalase-ethyl hydrogen peroxide complex with hydrogen peroxide (and ethyl hydrogen peroxide to avoid a dilution effect), the tracing of record D immediately rises, indicating a decrease of optical density which occurs in about 5 milliseconds after mixing; ethyl hydrogen peroxide is removed from catalase hematins in a very rapid chemical reaction with hydrogen peroxide. The tracing does not, however, rise to the optical density of free catalase; it only rises to the optical density of the catalase-hydrogen peroxide complex. Thus hydrogen peroxide strips catalase hematins of their ethyl hydrogen peroxide groups in a few milliseconds and at the same time combines with catalase hematin to form the hydrogen peroxide complex and thereby the hydrogen peroxide is decomposed in the ordinary catalatic reaction. The downward deflection of the platinum microelectrode tracing clearly shows that hydrogen peroxide is present in the observation tube during the flow but is all decomposed by catalase as soon as the flow stops; the activity of catalase does not differ significantly from record A and is not inhibited by the preformed catalase-ethyl hydrogen peroxide complex.

When the hydrogen peroxide has been decomposed, ethyl hydrogen peroxide can recombine with catalase, as indicated by the downward deflection of the spectrophotometer tracing after the flow stops.

The reaction of catalase ethyl hydrogen peroxide with hydrogen peroxide is about 90 per cent complete in a time of several milliseconds, which corresponds to a reaction velocity constant in excess of 10^6 M^{-1} $\times$ sec.$^{-1}$.

Reaction of Catalase Methyl Hydrogen Peroxide with Hydrogen Peroxide

The reaction of Fig. 1 can also be demonstrated for the catalase-methyl hydrogen peroxide complex, as is shown by Fig. 2. Record A is analogous to record B of Fig. 1 and gives the saturation value of the catalase-methyl

hydrogen peroxide complex. In records *B*, *C*, and *D*, catalase and 10 μM of methyl hydrogen peroxide are first mixed in a test-tube and are then mixed with a second 10 μM portion of methyl hydrogen peroxide, along with the other reactants noted in Fig. 2, in the rapid flow apparatus.

Record *B* is a control which indicates that catalase methyl hydrogen peroxide I formed in this test is stable over the required period. Since an excess of ethanol is present, the base-line from the previous injection of reactants into the capillary of the flow apparatus corresponds to catalase. On starting the flow, the capillary is filled with the preformed catalase-methyl hydrogen peroxide, an extra 10 μM of methyl hydrogen peroxide,
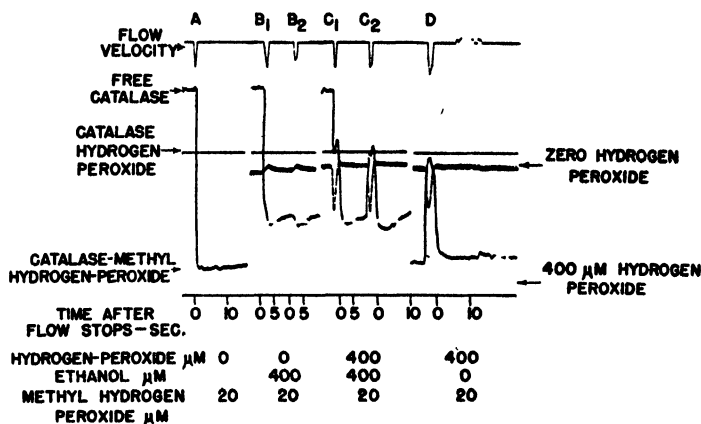
EXPT 154

FIG. 2. Reaction of catalase methyl hydrogen peroxide with hydrogen peroxide. In record *A*, 0.57 μM of horse liver catalase is mixed with the reactants listed beneath the record. In records *B*, *C*, and *D*, catalase methyl hydrogen peroxide is preformed and then mixed with the reactants listed. The kinetics of the complexes and of hydrogen peroxide are recorded as in Fig. 1. B_2 and C_2 represent a second discharge of the same reactants used in B_1 and C_1 respectively.

and ethanol. The complex is stable for several seconds, and, before appreciable decomposition has taken place, the flow is restarted at B_2, replacing the catalase methyl hydrogen peroxide that existed in the presence of a known excess of methyl hydrogen peroxide (10 μM) by the catalase methyl hydrogen peroxide in the storage syringe. Since there was no appreciable optical density change, the material in the storage syringe is sufficiently stable. Its maximum concentration is less than the saturation value, but this is because alcohol is present.

In records *C* and *D*, Fig. 2, the actual tests of the reaction of hydrogen peroxide and catalase-bound methyl hydrogen peroxide are made. Since

an excess of ethanol is present, record C starts with the optical density of free catalase. On injection of the preformed catalase methyl hydrogen peroxide, the optical density of catalase methyl hydrogen peroxide found in record B is not obtained during the flow; a downward deflection of about one-third that of record A is obtained, corresponding to the optical density of catalase hydrogen peroxide. As in Fig. 1, the platinum electrode indicates the presence of hydrogen peroxide in the capillary during the flow. As the flow stops, the optical density becomes equal to that of record B, owing to the reformation of the catalase-methyl hydrogen peroxide complex. The injection of fresh reactants several seconds later at C_2 gives a very large contrast to record B_2, for here the optical density immediately decreases to that of catalase hydrogen peroxide and is followed by the reformation of the catalase methyl hydrogen peroxide when the flow stops. This record is analogous to record D of Fig. 1. The half time for the decomposition of this complex ($t_{\frac{1}{2} \text{ off}}$) into free catalase is only about half that found in record B. This indicates that about one-half of the methyl hydrogen peroxide is destroyed in the brief interval required for the destruction of the hydrogen peroxide. Stern's early experiment (1) with catalase, hydrogen peroxide, and methyl hydrogen peroxide is thereby explained; not only does catalase methyl hydrogen peroxide react rapidly with hydrogen peroxide but also the excess methyl hydrogen peroxide recombines rapidly enough with catalase so that a considerable turnover of methyl hydrogen peroxide occurs simultaneously because of the reaction of this complex with hydrogen peroxide.

In record D, Fig. 2, ethanol is omitted, and it is seen that the optical density of the complex from the previous injection is equal to that of record A; catalase is saturated with methyl hydrogen peroxide. On mixing the complex with hydrogen peroxide, a similar rapid decrease of optical density to that of catalase hydrogen peroxide is seen. This indicates that the reaction may occur with partially or fully saturated catalase methyl hydrogen peroxide.

Only a small fraction of the catalase alkyl hydrogen peroxide is in the form of compound II in the records of Figs. 1 and 2. When the alkyl hydrogen peroxide concentration is increased to favor the formation of the secondary compounds, complete inhibition of catalase is not obtained in accordance with Stern's data (1). Since it is unlikely that the secondary compounds react with hydrogen peroxide, the activity of catalase in the presence of a large excess of alkyl hydrogen peroxide is probably caused by the reaction of a small amount of the primary compound with hydrogen peroxide in the manner shown here.

A much more vivid demonstration of the rapid reaction of hydrogen

peroxide with catalase methyl hydrogen peroxide I is afforded when the hydrogen peroxide is continuously generated, as by notatin, glucose, and oxygen (7). Here the reaction of hydrogen peroxide with catalase-bound methyl hydrogen peroxide continues until all the methyl hydrogen peroxide has disappeared. The velocity constant for the reaction of hydrogen peroxide with catalase methyl hydrogen peroxide is estimated to be about 5×10^7 $M^{-1} \times$ sec.$^{-1}$.

DISCUSSION

Inhibition of Catalase by Alkyl Hydrogen Peroxide—These experiments show that catalase, incubated with alkyl hydrogen peroxides long enough to form the primary complexes with all four catalase hematins bound, still reacts very rapidly with hydrogen peroxide. Thus no inhibition of catalase is caused by these primary complexes. However, in the presence of large amounts of ethyl hydrogen peroxide (0.13 N) as used by Stern (1), competitive inhibition of catalase has been demonstrated. We offer the following explanation based upon these experiments. Ethyl hydrogen peroxide (0.13 N) can react rapidly enough with catalase to form a small steady state concentration of the primary complex which will change to the secondary complex. The secondary complex formed in the cuvette of a recording spectrophotometer does not decompose on the addition of an excess of hydrogen peroxide (7). Thus catalase activity is inhibited by the inactive secondary catalase-ethyl hydrogen peroxide complex. Such inhibition will be competitive, as found by Stern, since the steady state concentration of the primary catalase-ethyl hydrogen peroxide complex will be decreased on increasing the hydrogen peroxide concentration. Thus the formation of the inactive secondary complex will be decreased. The competition is between hydrogen peroxide and catalase ethyl hydrogen peroxide II for catalase ethyl hydrogen peroxide I.

Reaction Mechanisms—The experiments just described provide an important link in the development of tentative reaction mechanisms for catalase which explain both the peroxidatic and catalatic[3] activities. In the equations which follow, the symbol Fe stands for any one of the four catalase hematin-iron groups. The hydroxyl group found by Agner and Theorell (8) is replaced by the peroxide group in these reactions (2). Possible intermediate steps in these reactions, such as the formation of free radicals, are omitted. The momentary formation of a catalase perox-

---

[3] Catalatic activity is here defined as the reaction of a hemoprotein with hydrogen peroxide, giving water and oxygen. Peroxidatic activity is here defined as the reaction of a hemoprotein with a peroxide molecule and an acceptor molecule in which the acceptor is oxidized and no oxygen is evolved.

ide and alcohol complex would make it possible for both the hydrogen atoms of the alcohol to be transferred simultaneously, as indicated in these equations.

It is possible that the acceptor molecule is attached to specific groups in the catalase protein in this complex. Since such groups are not yet known, this step in the reaction is omitted. The equations for the formation of the catalase-peroxide complexes and for their conversion into the secondary complexes were given previously (2); here only the reactions of the catalase peroxide molecules will be given.

The oxidation of alcohols by catalase-hydrogen peroxide is represented by Equation 1.

$$\text{Fe}\overset{\cdot\cdot}{O}\text{OH} \longleftrightarrow \text{FeOH} + \text{H}_2\text{O}$$
$$R-\overset{\overset{H}{|}}{\underset{\underset{H}{|}}{C}}-\text{OH} \qquad R-\overset{\underset{H}{|}}{C}=O \qquad (1)$$
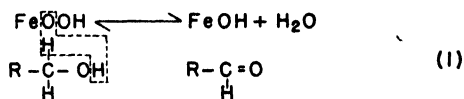
With alkyl hydrogen peroxides, the reaction is similar. The equations indicate the possibility that an alcohol is formed from the alkyl hydrogen peroxide.

$$\text{Fe}\overset{\cdot\cdot}{O}\text{OR} \longleftrightarrow \text{FeOH} + \text{ROH}$$
$$R-\overset{\overset{H}{|}}{\underset{\underset{H}{|}}{C}}-\text{OH} \qquad R-\overset{\underset{H}{|}}{C}=O \qquad (2)$$
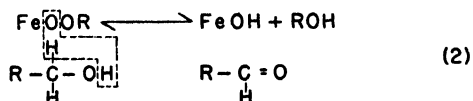
Here the choice of the reactive oxygen atom of the peroxide group is purely arbitrary.

The experiments of this paper show that hydrogen peroxide can replace an alcohol,

$$\text{Fe}\overset{\cdot\cdot}{O}\text{OR} \longleftrightarrow \text{FeOH} + \text{ROH}$$
$$\text{HOOH} \qquad \text{O}_2 \qquad (3)$$
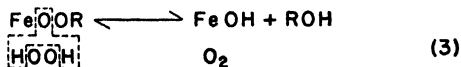
probably giving oxygen and an alcohol.

In Equations 1 and 2, the catalase-peroxide complex has been represented as a dehydrogenase which removes 2 hydrogen atoms from the acceptor molecule in accord with studies of peroxidase.[4]

There is strong support that the representation given in Equation 3 is correct; if the following reaction occurred,

$$\text{Fe}\overset{\cdot\cdot}{O}\text{OR} \longleftrightarrow \text{FeOH} + \text{ROH}$$
$$\text{HOOH} \qquad \text{O}_2 \qquad (4)$$
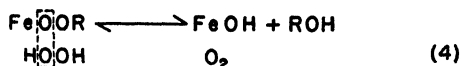
there is no reason why the analogous reaction should not occur,

[4] Chance, to be published.

$$Fe\overline{|O|}OR \longleftrightarrow FeOH + 2ROH$$
$$H_2O + H\underline{|O|}OR \qquad O_2 \qquad (5)$$
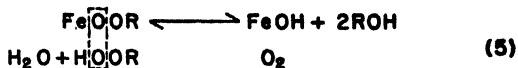
But the reaction of Equation 5 most certainly does not occur; the absence of oxygen evolution is an outstanding feature of the "spontaneous" decomposition of the catalase-alkyl hydrogen peroxides. Therefore, Equation 4 is extremely dubious, and Equation 3 has been adopted as correct.

The catalatic reaction, by analogy with Equation 3, is then

$$Fe\overline{|O|}OH \longleftrightarrow FeOH + H_2O$$
$$\underline{|H|O|O|H|} \qquad O_2 \qquad (6)$$
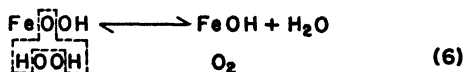
According to this mechanism, the catalatic activity is the special case of peroxidatic activity in which the substrate[5] and acceptor[6] molecules are the same. The peroxide molecule attached to the iron atom is reduced, and the 2nd peroxide molecule is oxidized.

The mechanism of Equation 6 does not indicate that horseradish and lactoperoxidases would act catalatically, for they show a high reactivity towards a related, but different, molecular structure of the acceptor,[4] for example the enediol group, and do not react with hydrogen peroxide as an acceptor.

An alternative mechanism, which is based upon none of the properties of catalase acting peroxidatically, is that substrate and acceptor molecules attach to different hematin groups of catalase (see Theorell (9)). Such a mechanism cannot apply to the reactions of the catalase alkyl hydrogen peroxides, since no inhibition of the reactions represented by Equations 2 and 3 is obtained when all the hematin groups of catalase are bound to alkyl hydrogen peroxide. Thus this mechanism is applicable only to the catalatic reaction and may be represented by Equation 7.

$$
\begin{array}{c}
\text{HOFe—FeOOH} \\
|\quad\quad| \qquad + \text{HOOH} \longleftrightarrow \\
\text{HOFe—FeOH}
\end{array}
\begin{array}{c}
\text{HOFe—FeOOH} \\
|\quad\quad| \qquad\longleftrightarrow \\
\text{HOFe—FeOOH}
\end{array}
$$

$$+ \text{H}_2\text{O}$$
$$
\begin{array}{c}
\text{HOFe—FeOH} \\
|\quad\quad| \qquad + \text{O}_2 \qquad (7) \\
\text{HOFe—FeOH}
\end{array}
$$
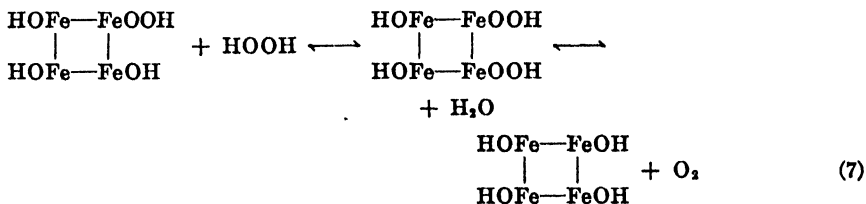
When the 2nd hydrogen peroxide molecule becomes attached to any one of the free hematin groups to form a hypothetical complex, 2 electrons

[5] The substrate molecule is here defined as the molecule that participates in the enzyme-substrate complex and that is reduced on reaction with the acceptor molecule.

[6] The acceptor molecule is here defined as the molecule which is oxidized by the enzyme-substrate complex and is so named here because it accepts the oxygen atom of the peroxide group.

are transferred internally through the catalase protein from one of the hydrogen peroxide molecules to the other. Thus this is termed an "internal" reaction.[7] The life time of the catalase complex with 2 peroxide molecules attached must be very short to agree with the experimental data.

According to Equation 7, catalase catalyzes the destruction of hydrogen peroxide by virtue of having at least two hematin groups attached to the same protein molecule, while only one hematin group is required by Equation 6. Equation 7 is attractive because it provides a simple explanation of why horseradish and lactoperoxidases do not cause the catalatic reaction: they have only one hematin group. This explanation also includes the 4-hematin methemoglobin because there only secondary inactive peroxide complexes are formed. Equation 7 also provides a simple explanation of the decrease of catalase activity with the decrease of the number of intact hematin groups. Bonnichsen (10) found that a 2.3-hematin catalase was considerably less than half as active as a 4-hematin catalase. The serious drawback of Equation 7 is that it cannot apply to the data given in this paper and in that of Chance (7) on the reaction of catalase alkyl hydrogen peroxides with hydrogen peroxide.

The composition of catalase hydrogen peroxide of 1.2 ± 0.1 peroxide-bound hematins per catalase molecule is explained on the basis of the consecutive reactions of hydrogen peroxide with catalase and with catalase hydrogen peroxide. In Equation 6, this means that each hematin is 30 per cent saturated with hydrogen peroxide, while in Equation 7 each *pair* of hematins is 30 per cent saturated with hydrogen peroxide. If the total number of hematins in catalase is reduced to two, then the composition of catalase hydrogen peroxide according to Equations 6 and 7 would be 30 per cent $\times$ 2 or equivalent to 0.6 hematin group. But no such change in the composition of catalase hydrogen peroxide has been found with a 2.7-hematin catalase. Therefore, heme-heme interaction has been postulated for which there is no evidence from the studies of either the cyanide or alkyl hydrogen peroxide complexes.

A rather different theory, which does not require a postulation of heme-heme interaction, is represented by Equation 8 in which one of the catalase hematins is fully saturated with hydrogen peroxide, and, because of this, the whole catalase molecule acquires special properties and is able to decompose hydrogen peroxide directly into free radicals.

$$\begin{matrix} \text{HOFe—FeOOH} & & \text{HOFe—FeOOH} & \\ \mid \quad \mid & + \text{ HOOH} \longrightarrow & \mid \quad \mid & + \text{ 2OH} \qquad (8) \\ \text{HOFe—FeOH} & & \text{HOFe—FeOH} & \end{matrix}$$

---
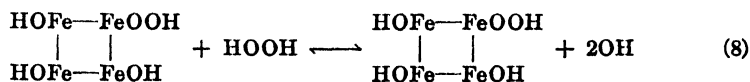
[7] An internal reaction is defined as one in which the reactants attach to different hematin groups on the same catalase molecule, and the reaction occurs by electron transfer through the catalase molecule.

The attachment of the 2nd peroxide molecule could be either to the peroxide-bound hematin or to a free catalase hematin. This theory, however, requires that the composition of catalase hydrogen peroxide be 1.0 peroxide-bound hematin per catalase molecule (see Chance (7)).

The mathematical solutions for the kinetics of Equations 6, 7, and 8 are being studied and give very nearly identical results over the range of experimental conditions that can now be investigated and as yet give no preference to any one of the three mechanisms for the catalatic reaction. However, the mechanism of Equation 6 is much preferred because it applies to both the peroxidatic and catalatic reactions.

A general equation for catalase action which covers Equations 6 and 7 has been proposed previously (for a summary, see Sumner (11)).

$$Fe \cdot OH + H_2O_2 \longrightarrow Fe \cdot OOH + H_2O \tag{9}$$

$$Fe \cdot OOH + H_2O_2 \longrightarrow Fe \cdot OH + H_2O + O_2 \tag{10}$$
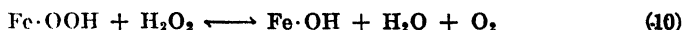
The speculation involved in these equations now receives support because of the following findings: (1) the intermediate compound of catalase and hydrogen peroxide (6); (2) the lack of a Michaelis constant for catalase action (12); (3) the peroxidatic reactions of catalase hydrogen peroxide (6). It is interesting to note that none of the sponsors of these equations realized that these consecutive reactions would not give the then accepted Michaelis constant of catalase, which has now been proved to be an artifact due to catalase inactivation (12).

An up to date form of the equations is

$$Cat(OH)_4 + H_2O_2 \xleftarrow{\sim 3 \times 10^7 \, M^{-1} \times sec.^{-1}} Cat(OH)_3OOH + H_2O \tag{11}$$

$$Cat(OH)_3OOH + H_2O_2 \xleftarrow{\sim 3.5 \times 10^7 \, M^{-1} \times sec.^{-1}} Cat(OH)_4 + H_2O + O_2 \tag{12}$$
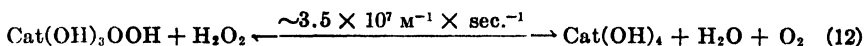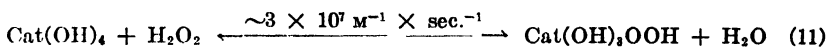
where the symbol Cat represents the catalase molecule.

In the mechanism recently proposed by Lemberg and Foulkes (13) for the catalatic and peroxidatic activities, the catalase-hydrogen peroxide complex spontaneously breaks down into oxygen and $(XH_4)Fe^{III}$ and then reacts with a 2nd hydrogen peroxide molecule or with an alcohol. But the lack of oxygen evolution in the peroxidatic activity of catalase is one of the fundamental features of Keilin and Hartree's experiments on coupled oxidations (14).

Substrate and Acceptor Specificity of Catalase—The lack of activity of catalase with diethyl peroxide (1) shows that substrates of the type R—OOH are required to form the catalase-peroxide complexes. In catalatic activity in which the substrate also serves as acceptor, 2 hydrogen atoms are required of the peroxide molecule, and only hydrogen peroxide is suitable.

Catalase peroxides oxidize methanol, methylene glycol, and formate

with about equal velocity, and all these molecules have a hydrogen atom and a hydroxyl group attached to carbon: $H-\overset{|}{\underset{|}{C}}-OH$ (2). The catalase-peroxide complexes act as a dehydrogenase and remove 2 hydrogen atoms from this group. Of the analogous compounds, ethanol, acetaldehyde, and acetic acid, only ethanol, which has the $H-\overset{|}{\underset{|}{C}}-OH$ group, is oxidized. Thus both the hydroxyl group and the hydrogen atom must be attached to the same carbon atom. The acceptor action of hydrogen peroxide indicates that the carbon atom may be replaced by oxygen in hydrogen peroxide.[8] Replacement of the hydroxyl oxygen atom by sulfur as in ethyl mercaptan results in a much slower reaction velocity, about one-tenth.

The reactivity of catalase peroxides towards large molecules having the $H-\overset{|}{\underset{|}{C}}-OH$ group is severely restricted by the limited accessibility of catalase hematin, and many substances of biological importance which contain this group have been found to be too large to react with catalase with a velocity comparable to that obtained with the small molecules.

The experiments of Keilin and Hartree (14), in which hemoglobin is oxidized to methemoglobin in the presence of catalase and the notatin system, is not a coupled oxidation in which hemoglobin is an acceptor; the reaction proceeds farther in the absence of catalase.

SUMMARY

1. Hydrogen peroxide reacts very rapidly with the primary catalase-alkyl hydrogen peroxide complexes in a fashion analogous to their peroxidatic reactions with alcohols. Thus hydrogen peroxide can act as an acceptor. The reaction of hydrogen peroxide with the catalase-hydrogen peroxide complex is considered to occur in the same fashion; here hydrogen peroxide molecules act as substrates or acceptors.

2. The primary catalase-alkyl hydrogen peroxide complexes are not inhibitors of the catalatic activity; the competitive inhibition found by Stern is therefore attributed to the formation of the secondary catalase-ethyl hydrogen peroxide complex.

3. Catalase requires a hydrogen atom in the peroxide group (HOOR) acting as a substrate and a hydrogen atom and a hydroxyl group

[8] The recent work of Heppel and Porterfield (15) shows that catalase oxidizes nitrite in coupled oxidations. We confirm their results by these methods and we may include nitrogen along with carbon and oxygen.

$\overset{\displaystyle |}{(\text{H}\!-\!\overset{\displaystyle |}{\underset{\displaystyle |}{\text{C}}}\!-\!\text{OH})}$ attached to a carbon atom in the alcohol or acid acting as an acceptor.

4. Catalatic activity requires the participation of 2 molecules of hydrogen peroxide. The 1st molecule is required to form the catalase-hydrogen peroxide complex. Three mechanisms are discussed for the mode of action of the 2nd peroxide molecule: (1) it may react near the peroxide group already attached to catalase hematin (a peroxidatic mechanism); (2) it may react with a free catalase hematin (an internal reaction) to give free catalase, oxygen, and water; or (3) it may react to give catalase hydrogen peroxide and free radicals. No final decision can yet be reached on the exact mechanism of reaction of the 2nd peroxide molecule.

BIBLIOGRAPHY

1. Stern, K. G., Z. physiol. Chem., 209, 176 (1932).
2. Chance, B., J. Biol. Chem., 179, 1341 (1949).
3. Chance, B. J. Biol. Chem., 179, 1311 (1949).
4. Chance, B. J. Biol. Chem., 179, 1299 (1949).
5. Chance, B. Rev. Scient. Instruments, 18, 601 (1947).
6. Chance, B. Acta. chem. Scand., 1, 236 (1947).
7. Chance, B. Biochem. J., in press.
8. Agner, K., and Theorell, H., Arch. Biochem., 10, 321 (1946).
9. Theorell, H., Experientia, 4, 100 (1948).
10. Bonnichsen, R. K., Dissertation, Karolinska Institutets, Stockholm (1948).
11. Sumner, J. B., Advances in Enzymol., 1, 170 (1941).
12. Bonnichsen, R. K., Chance, B., and Theorell, H., Acta. chem. Scand., 1, 685 (1947).
13. Lemberg, R., and Foulkes, E. C., Nature, 161, 131 (1948).
14. Keilin, D., and Hartree, E. F., Biochem. J., 37, 293 (1945).
15. Heppel, L. A., and Porterfield, V. T., J. Biol. Chem., 178, 549 (1949).

SERUM VASOCONSTRICTOR (SEROTONIN)

V. THE PRESENCE OF CREATININE IN THE COMPLEX. A PROPOSED STRUCTURE OF THE VASOCONSTRICTOR PRINCIPLE

By MAURICE M. RAPPORT

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York)

In the fourth paper in this series (1) the isolation of the substance believed to be responsible for the marked vasoconstrictor activity of serum was reported. This substance was characterized as an indole derivative from its ultraviolet absorption spectrum and its behavior in various color and other chemical reactions. The analytical data agreed well with the empirical formula $C_{14}H_{21}O_3N_5 \cdot H_2SO_4$. The titration curve in aqueous solution revealed two titratable groups with pK'_1 of 4.9 and pK'_2 of 9.8. Both of these groups were believed to be basic in nature, and the pK' value of the weaker suggested an acylated guanidine structure. Indeed, the value was very close to that reported in the literature for creatinine, while the formation of ammonia on treatment with alkali and the presence of one N-methyl grouping suggested that a creatinine residue might well be present in the molecule.

The fact that only exceedingly small quantities of pure substance were available led to an investigation of this possibility by colorimetric means, and it was quickly determined that serotonin sulfate responded positively to both the Jaffe alkaline picrate reagent and the Benedict alkaline 3,5-dinitrobenzoic acid reagent, with a color intensity equal (within an experimental error of about 10 per cent) to that of creatinine on a molar basis. Benedict's study (2) of the specificity of the alkaline 3,5-dinitrobenzoic acid reagent with respect to creatinine showed that derivatives and closely related compounds such as glycocyamidine could be readily distinguished by measuring both the intensity of color and the rate of color development. Comparison of serotonin sulfate with creatinine in this fashion again revealed almost identical behavior.

It then became necessary to investigate the question of whether a creatinine-like residue was actually part of the molecular structure of serotonin, or whether, as now seemed more likely, the entity isolated and designated as serotonin sulfate was not in reality a complex containing an indole base, creatinine, and sulfuric acid.

The behavior of serotonin on a paper chromatogram developed with butanol showed that the latter was indeed the case, for the creatinine chromogen was completely separable from the indole chromogen.

The presence of creatinine was then directly confirmed by isolation of its picrate. In addition, the crystalline picrate of the indole base was obtained for the first time. Elementary analysis of the latter confirmed the empirical formula $C_{10}H_{14}O_2N_2$ obtained by deducting that of creatinine $(C_4H_7ON_3)$ from that of the complex $(C_{14}H_{21}O_3N_5)$.

EXPERIMENTAL

Melting points were determined on the micro hot stage and are uncorrected.

Color Reactions—The serotonin sulfate complex was compared with creatinine for intensity of color per mole in both the alkaline dinitrobenzoic acid reaction of Benedict and the alkaline picric acid reaction of Jaffe.

The method used for the dinitrobenzoic acid reaction was that described by Benedict and Behre (2). To 5.0 ml. of unknown in aqueous solution were added 1.0 ml. of 5 per cent ethanolic 3,5-dinitrobenzoic acid and then 1.0 ml. of 5 per cent aqueous sodium hydroxide. After standing 15 minutes at room temperature, the optical density was determined at $\lambda = 5400$ A with a Coleman model 11 universal spectrophotometer and No. 6-304B cuvettes.

The picric acid reaction was carried out as follows. To 5.0 ml. of unknown solution were added 2.5 ml. of an alkaline picric acid solution made by adding 1.0 ml. of 10 per cent aqueous sodium hydroxide to 5.0 ml. of saturated aqueous picric acid. After standing at room temperature for 20 minutes, the color was determined with the Coleman instrument at $\lambda = 5200$ A.

The results (Table I) show that serotonin complex gives a molar color development almost equal to that of creatinine by both methods.

The rates of color formation with Benedict's reagent of serotonin sulfate complex and creatinine (Table II) are almost identical. In this experiment the ratio of color per mole of serotonin complex to color per mole of creatinine is 0.94.

Paper Chromatography—40 γ of serotonin sulfate complex in 0.01 ml. of water and 10 γ of creatinine in 0.01 ml. of water were placed on Whatman No. 1 filter paper and developed with butanol saturated with water by downward irrigation for 17 hours at room temperature. Two strips of the serotonin and one of the creatinine were run simultaneously. After drying in air, one of the serotonin strips (C) was cut off and sprayed with Ehrlich's reagent.[1] The remaining two strips $(A$ and $B)$ were

---

[1] The spraying reagent was prepared by adding 6 ml. of butanol to 1 ml. of a solution containing 1 gm. of p-dimethylaminobenzaldehyde in a mixture of 30 ml. of 95 per cent ethanol and 30 ml. of concentrated hydrochloric acid (5).

sprayed with Benedict's reagent.[2] The results (Fig. 1) show that the indole portion of the complex (C) was completely separated from the creatinine portion (B) and that the latter migrates with almost the same velocity (R_F = 0.15) as creatinine (R_F = 0.16). The colors obtained

TABLE I

Comparison of Intensity of Color Produced by Serotonin Sulfate Complex and Creatinine in Benedict and Jaffe Reactions

	Benedict alkaline 3,5-dinitrobenzoate		Jaffe alkaline picrate	
	Serotonin sulfate complex, 33 γ	Creatinine, 10 γ	Serotonin sulfate complex, 41 γ	Creatinine, 10 γ
Color density	0.033	0.039	0.052	0.048
Color per mole serotonin sulfate complex / Color per mole creatinine	0.92		0.95	

* The quantity of serotonin sulfate complex was determined from the ultraviolet absorption at 2750 A in the Beckman spectrophotometer, with 10 mm. cells. The calculation was made from the formula, γ per ml. = (density × 100)/1.43, for densities between 0.2 and 0.6.

TABLE II

Rate of Color Development with Benedict's Reagent of Serotonin Sulfate Complex and Creatinine

Time	Serotonin sulfate complex, 205 γ		Creatinine, 50 γ	
	Color density	Per cent maximum color	Color density	Per cent maximum color
min				
1	0.084	36	0.079	37
3	0.148	64	0.139	65
5	0.182	78	0.169	79
10	0.218	94	0.203	94
15	0.231	100	0.215	100
20	0.232	100	0.215	100
30	0.220	95	0.196	91

* See foot-note to Table I.

with both Ehrlich's and Benedict's reagents are initially violet. The color developed with Benedict's reagent fades to pink and then disap-

[2] The spraying reagent was prepared by adding 3 ml. of butanol to a solution of 2 ml. of 5 per cent ethanolic dinitrobenzoic acid plus 1 ml. of 5 per cent aqueous sodium hydroxide.

pears. A pale brown color slowly develops at the position of the indole base (strip B). This reaction is believed to be responsible for the less rapid fading of the color obtained with serotonin sulfate complex as compared with creatinine (Table II).

Isolation of Picrates—9.4 mg. of serotonin sulfate complex were dissolved in 0.5 ml. of water by slight warming and 0.5 ml. of saturated aqueous picric acid was added. A faint reddish coloration and the development of turbidity were observed. The solution was warmed until it became almost clear, and it was then allowed to cool slowly. Beautiful orange-red needles began to separate as crosses which became tufted and then developed through sheaves into perfect rosettes. On standing
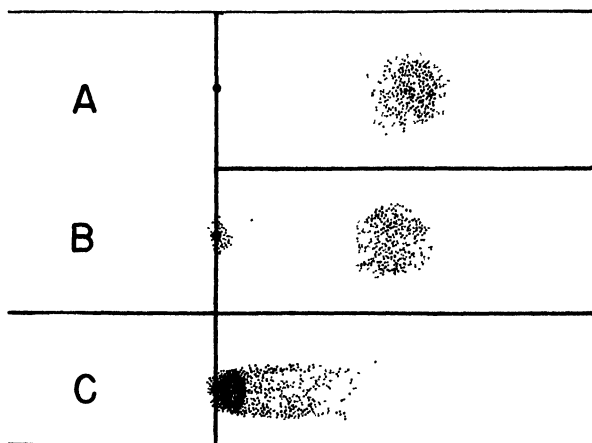
FIG. 1. Paper chromatogram showing the separation of creatinine from the indole portion of serotonin sulfate complex. A, 10 γ of creatinine, B, 40 γ of serotonin complex developed with creatinine reagent, C, 40 γ of serotonin complex developed with Ehrlich's reagent. For details see the text.

overnight a second kind of crystal made its appearance, dense yellow rosettes of prisms. The crystals were centrifuged, washed with a minimum quantity of water, and dried by evaporation on the water pump. The four dense yellow clusters (3.5 mg.) were separated mechanically from the fluffy orange-red needles (4.3 mg.).

The yellow clusters were recrystallized from a small quantity of water and separated as individual stout yellow prisms melting with decomposition at 207–210° when heated at a rate of 2° per minute. Creatinine picrate was prepared from an authentic sample of creatinine (Pfanstiehl) and was obtained on recrystallization from water as typical, long, straw-like, yellow needles which melted, under identical conditions, at 208–211° with decomposition. The mixed melting point was 207–210°.

In a qualitative spot test with Benedict's reagent, both samples showed identical behavior, forming an orange-red color at the site of the crystal which developed into a violet-brown color in the solution.

It is believed that the evidence favoring the identity of the two picrates outweighs the observed difference in crystal habit.

The orange-red needles of the indole picrate were recrystallized twice from water. The melting point determination was complicated by several distinct transitions, unaffected by further recrystallization. Fusion began at 105–110°. Some crystals melted completely, but the mass slowly became deep red in color. Resolidification of the melted crystals then began and was essentially complete by 130°. The deep red color
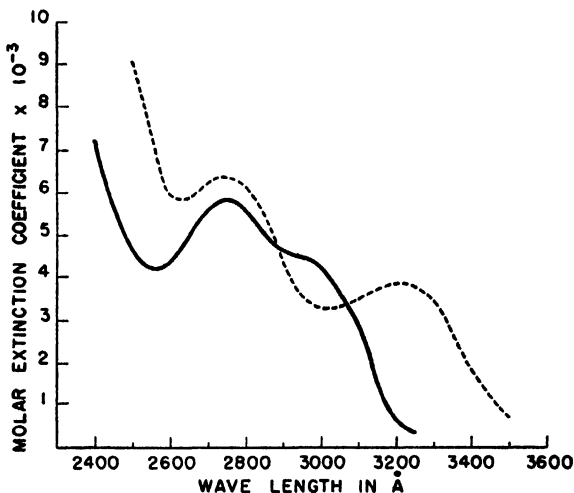
FIG. 2. Ultraviolet absorption spectrum of serotonin in water at pH 5.4 (solid line) and pH 11.6 (dotted line).

paled from 165–175°, and complete fusion with decomposition occurred at 185–188°. The rate of heating during transitions and melting was 2° per minute.

These crystals gave no color with Benedict's reagent, but with Ehrlich's and Folin's reagents qualitative spot tests were positive. The analytical sample was dried at 80° over phosphorus pentoxide *in vacuo* to constant weight.[3]

$C_{10}H_{14}O_2N_2 \cdot C_6H_3O_7N_3$. Calculated. C 45.39, H 4.05, N 16.55
423.3   Found.   " 45.65, " 4.23, " 16.95

[3] Analyses by Dr. A. Elek, Los Angeles.

Ultraviolet Absorption Spectrum—The ultraviolet absorption spectrum of serotonin sulfate complex was reported (1) to be essentially the same with regard to maxima and general contour in aqueous solution at pH 3.5 and 10.3. It has now been found that at pH 11.6 (Beckman pH meter) the absorption spectrum shows a remarkable shift of the second maximum (Fig. 2). The significance of this change will be discussed below. Absorption measurements were made with a Beckman model DU quartz spectrophotometer.

<div align="center">DISCUSSION</div>

The identification of creatinine in the serotonin sulfate complex confirms the molecular weight of 405 for this complex, since the dissociation constant characteristic of this substance is in accord with that obtained from the theoretical dissociation curve calculated on the assumption of this molecular weight (1). Additional evidence favoring this value is obtained from the molecular extinction at 2750 A which agrees well with that expected for absorption of indoles. There is, then, little doubt that the empirical formula of the indolic base portion of the complex (which is also, presumably, responsible for the pharmacological activity) is $C_{10}H_{14}O_2N_2$. The molecular complexity of the vasoconstrictor principle is thus not much greater than that of epinephrine.

If we subtract the formula for the indolyl group (C_8H_6N), the number of atoms which must still be put into place is very few, $C_2H_8O_2N$. The number of suitable structures which can be written is therefore quite limited, and before discussing the possibilities, it might be well to recapitulate those properties which an acceptable structure must satisfactorily explain.

The most important of these is the ultraviolet absorption spectrum which deviates in contour quite markedly from that found for such other widely occurring indoles as tryptophan, tryptamine, and indican. The shift in the second maximum from 2930 A to 3230 A which occurs on raising the pH to 11.6 (Fig. 2) and which is not observed at pH 10.3 (1) suggests the presence of a weakly acidic function. The position of the maximum at 2750 A, which remains unchanged during these shifts in pH, is consistent with indolic absorption. Clearly then, the maximum at 2930 A is attributable to a second absorbing group. It would be difficult to locate such a group in a side chain which maximally contains $C_2H_8O_2N$, especially since this empirical formula is already above the theoretical degree of saturation. The assumption of a second nuclear substitution of the indole must therefore be made, and a phenolic grouping would most easily account for the observed facts. This conclusion gains further support from the color reaction with the Folin-Ciocalteau reagent,

which indicates the presence of a second reducing group besides the indole (1). It might be remarked that several attempts to detect the odor of indole on micro zinc dust distillation of serotonin sulfate complex met with no success, although positive results were easily obtainable with compounds containing a simple indole nucleus.

With regard to the location of this phenolic group, the intensity and rate of color development in the quantitative Hopkins-Cole reaction (1) indicate that positions 1 and 2 of the indole nucleus are unsubstituted. Comparison of both the Hopkins-Cole reaction and the ultraviolet absorption with that of indican[4] rules out position 3. Of the remaining four positions in the benzene ring, biological analogy to such naturally occurring compounds as physostigmine and, more importantly, bufotenine (I), strongly favors position 5. As has been pointed out by Wieland *et al.* (3), no simple indole substituted in positions 4 or 7 has ever been found in nature. Although these monohydroxyindoles have never been synthesized,[5] the methoxy compounds are known. Pruckner and Witkop (4) have published the ultraviolet absorption spectra of 5-methoxy- and 6-methoxyindole dissolved in alcohol, in connection with their studies of yohimbine. The curves are quite different from each other, and that of the 5-methoxy compound shows the same contour and almost the identical molecular extinctions of the two maxima observed with serotonin. Although the positions of the maxima of 5-methoxyindole are displaced 60 A toward the shorter wave-lengths, the similarities lend considerable weight to the argument favoring the 5 position for the phenolic substituent.

If the assumptions made are correct, the structure would now stand at II. It can be readily seen that two possibilities can explain such an

(I)   R = $CH_2CH_2N(CH_3)_2$
(II) R = C_2H_6ON
(III) R = $CH_2N(CH_3)_2$
(IV) R = $CH_2CH_2NH_2$
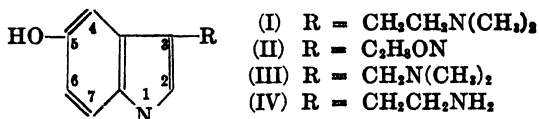
anomalous side chain. The first would require that the empirical formula be in error by two hydrogen atoms, and the second would postulate the presence of 1 mole of water of crystallization. With regard to the first, the three analyses are consistent in showing hydrogen values which are higher than the theoretical. An excess of two hydrogen atoms in the empirical formula would therefore mean that the analytical results were

[4] Unpublished experiments.

[5] The synthesis of the monohydroxyindoles has been described in a recent paper (Beer, R. J. S., Clarke, K., Khorana, H. G., and Robertson, A., *J. Chem. Soc.*, 1605 (1948)).

in error for hydrogen by 12 to 18 per cent, a possibility which seems unlikely.

The second alternative, namely, the presence of water of crystallization, is believed to be more consistent with the facts. Although such hydrate water was not detectable in the complex despite prolonged drying at 110°,[3] hydrates which do not lose their water below the decomposition point are known to occur. In fact, with the compound most closely related to that under consideration, namely, bufotenine (I), this phenomenon led to an incorrect empirical formulation on the basis of analyses of the oxalate salt (3). Inasmuch as both serotonin derivatives which were analyzed were crystallized from water, the probability of the presence of hydrate water is high. It might be noted further that the sulfate complex effervesces slightly on decomposing (1), while the picrate shows a decided transition at 105–110°, both observations being explainable by the presence of hydrate water.

If this assumption is made, the side chain would be C_2H_6N. With the exception of the presence of a basic group dissociating with a pK' of 9.8 (1), there is no evidence pertaining to this grouping. However, analogy to those compounds known to exert such powerful pharmacological effects presents an exceptionally strong argument in favor of a simple ethylamine side chain. Tentatively, then, the constitutional formula of 5-hydroxy-tryptamine (IV) may be assigned to this vasoconstrictor principle isolated from beef serum.

It should be mentioned that, besides the necessity for assuming the presence of water of crystallization, one other property of the substance is not in accord with the proposed structure, and that is the failure to obtain a red or violet color with ninhydrin-sodium acetate. This reaction is positive with tryptamine and most other primary amines. However, the evidence against a *secondary* amine formed from the available atoms is much stronger, namely, the presence of only 1 *N*-methyl group in the complex (1), which must be attributed to the creatinine, and the rate and intensity of color formation in the Hopkins-Cole reaction. The color development is exceptionally slow and the intensity very weak with gramine (III),[4] but they are comparable to that of tryptophan with serotonin.

Since a chemical method for the specific determination of serotonin is, at present, one of the most pressing problems connected with its further study, attention may be called to a property characteristic of this substance which may well serve as the basis for such a method. As can be seen from Fig. 2, the absorption of light at 3230 A increases sharply on raising the pH to 11.6. The location of this maximal increase, displaced as it is from the position of protein and other commonly encountered absorptions, as well as its dependence on pH should make it possible to determine serotonin specifically in amounts greater than 10 γ.

In the interest of simplicity, a further revision in nomenclature is proposed. The trivial name *serotonin* should be reserved for the pharmacologically active indole base. The complex would then be serotonin creatinine sulfate, and the second derivative, reported in this paper, serotonin picrate.

The serotonin creatinine sulfate used in this study was isolated in the laboratories of the Cleveland Clinic Foundation. The author wishes to express appreciation to Dr. Irvine H. Page for permission to retain this material. Grateful acknowledgment is also made to Dr. Hans T. Clarke for helpful discussions and assistance with the preparation of this manuscript.

SUMMARY

The crystalline vasoconstrictor substance isolated from beef serum has been shown to be a complex composed of equimolar parts of creatinine, sulfuric acid, and an indole derivative. The latter was converted into its picrate, analysis of which confirmed the empirical formula $C_{10}H_{14}O_2N_2$ obtained by deducting creatinine $(C_4H_7ON_3)$ from the formula of the complex $(C_{14}H_{21}O_3N_5)$. Evidence based on color reactions and ultraviolet absorption spectra indicates the presence of a 5-hydroxyindole nucleus in the structure. The constitutional formula of 5-hydroxytryptamine $(C_{10}H_{12}ON_2)$ has been tentatively proposed for this active principle.

A recommendation has been made to reserve the name *serotonin* for this indole amine rather than the previously isolated complex.

The basis for a simple specific method to determine serotonin by ultraviolet absorption spectrophotometry has been suggested.

BIBLIOGRAPHY

1. Rapport, M. M., Green, A. A., and Page, I. H., *J. Biol. Chem.*, **176**, 1243 (1948).
2. Benedict, S. R., and Behre, J. A., *J. Biol. Chem.*, **114**, 515 (1936).
3. Wieland, H., Konz, W., and Mittasch, H., *Ann. Chem.*, **513**, 1 (1934).
4. Pruckner, F., and Witkop, B., *Ann. Chem.*, **554**, 127 (1943).
5. Partridge, S. M., *Biochem. J.*, **42**, 240 (1948).

FORMATION OF CREATINE IN VITRO FROM METHYL PHOSPHATE AND GUANIDOACETIC ACID*

By FRANCIS BINKLEY and JEAN WATSON

(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Pathology and Medicine, University of Utah College of Medicine, Salt Lake City)

Methyl phosphate has been studied as a possible donor of methyl groupings for the formation of creatine by homogenates of liver tissue of rats and as a substitute for choline in the growth of rats receiving homocystine as the source of sulfur in their diet. It was found that methyl phosphate was an apparent source of methyl groupings in the *in vitro* synthesis of creatine but could not be substituted for choline in the diet of rats.

EXPERIMENTAL

Purification of Methyl Phosphate —The commercial product, methyl-phosphoric acid, was neutralized (to phenolphthalein) with sodium hydroxide to make an approximately 10 per cent solution of the sodium salt, and the material was precipitated by the addition of 3 volumes of ethanol. The crude precipitate was dissolved with boiling in the minimum amount of water, filtered, and allowed to cool. After three recrystallizations the material was free of inorganic phosphate. The total phosphorus was found to be 13.6 per cent (calculated for $Na_2CH_3PO_4 \cdot 4H_2O$, 13.6 per cent).

Formation of Creatine from Guanidoacetic Acid—All experiments were conducted in Warburg flasks in an atmosphere of N_2-CO_2 or O_2-CO_2 (5 per cent of CO_2 in each case). The buffer used was 0.06 M sodium bicarbonate. All materials were dissolved in bicarbonate or were neutralized by passing carbon dioxide through the solution. Homogenates of liver tissue were prepared by grinding an iced fresh liver of a rat with 5 volumes of the bicarbonate solution. 0.5 ml. of homogenate was used in all cases. Creatine was converted to creatinine and determined by the alkaline picrate reaction either directly on a trichloroacetic acid filtrate or by the procedure of Borsook and Dubnoff (1). Higher values were found by the latter procedure but better reproducibility could be obtained by the direct method. In Table I, a typical study is illustrated. It is seen that the formation of creatine is much greater under anaerobic con-

ditions and is augmented in the presence of citrate. Since citrate augmented the synthesis of creatine, the effect of magnesium ions was tested; it was found that the addition of magnesium ions greatly reduced the synthesis of creatine. As is illustrated in Table II, the effect of magnesium ions was probably due to an activation of the hydrolysis of methyl

TABLE I

Formation of Creatine from Guanidoacetic Acid and Methyl Phosphate

The creatine values, in every case, are the increment after 1 hour of incubation compared with a zero time digest prepared in exactly the same manner. 10 mg. of $Na_2CH_3PO_4 \cdot 4H_2O$ and 2 mg. of guanidoacetic acid were present, as indicated, in a total volume of 3.5 ml.

Digest	Activator	Creatine formed	
		N_2-CO_2	O_2-CO_2
		mg.	*mg.*
Homogenate..		0.00	0.00
" + guanidoacetic..		0.00	0.00
" + methyl phosphate.......		0.01	0.00
" + guanidoacetic + methyl phosphate		0.12	0.01
Same	Citrate (0.01 M)	0.28	0.02
"	$MgCl_2$ (0.001 ")	0.03	0.04

TABLE II

Hydrolysis of Methyl Phosphate and Formation of Creatine

The conditions were the same as in Table I.

Digest	Activator	Creatine formed	Inorganic phosphate
		mg.	*mg. P*
Homogenate......		0.00	0.03
" + methyl phosphate......		0.00	0.29
" + " " + guanidoacetic acid		0.10	0.21
Same	Citrate (0.01 M)	0.23	0.02
"	$MgCl_2$ (0.001 ")	0.02	0.73

phosphate. There was, of course, a corresponding increase in the carbon dioxide released from the buffer.

In Table III, a comparison of the activities of a homogenate and of an extract of liver tissue is given. The extract was prepared from the homogenate by high speed centrifugation. It is seen that an extract is active in the formation of creatine from guanidoacetic acid and methyl phosphate.

Feeding Experiments—Methyl phosphate was given to growing white rats in lieu of choline. The diet contained amino acid mixture (2) 27.4 gm., DL-homocystine 0.6 gm., cod liver oil 5.0 gm., sucrose 40.0 gm., and Spry 27.0 gm., and the water-soluble vitamins were given as described earlier (2). DL-Methionine, when added, was given in lieu of homocystine at the level of 0.8 gm. per 100 gm. of diet. Methyl phosphate was given as a neutralized solution at the rate of 100 mg. per day. In the experi-

TABLE III

Comparison of Homogenate and Extract in Formation of Creatine

Conditions as in Table I; N_2-CO_2 atmosphere with methyl phosphate and guanidoacetic acid in each digest.

Digest		0.01 M citrate	Creatine formed
			mg.
Homogenate	....	—	0.14
Extract.. .	. .. .. .	—	0.09
Homogenate.. .	. ..	+	0.21
Extract . ..	. .	+	0.17

TABLE IV

Growth of Rats Receiving Methyl Phosphate

Animal No.		Initial weight	Days	Addition to diet	Food consumption	Growth
		gm.			*gm. per day*	*gm. per day*
B1	♀	61	1–12	Methionine	4.7	+3.2
B3	♂	69	1– 5	"	4.9	+4.0
			6–12	" + methyl phosphate	4.7	+1.0
B5	♀	70	1–12	Choline	4.1	+1.7
			13–18	None	3.5	+0.2
			19–24	Methyl phosphate	3.0	−1.6
B7	♀	65	1– 5	None	3.2	−1.0
			6–12	Methyl phosphate	4.1	−0.2
			12–18	" " (injected)	4.0	−1.1

ments in which the methyl phosphate was injected a neutralized sterile solution was injected intraperitoneally. This study is summarized in Table IV. A litter of eight rats was used and the results with the second animals did not differ significantly from the animals reported here.

It is apparent from these studies that methyl phosphate will not substitute for choline in the growth of rats receiving homocystine as their source of sulfur. There was an indication that methyl phosphate in the diet was quite toxic; the growth of animals receiving methionine was depressed

by the administration of methyl phosphate.   Methyl phosphate, however, exerts a peculiar acute toxicity when injected intraperitoneally.   As little as 2 mg. of the material was sufficient to render the animals comatose. The animals recovered from doses as large as 25 mg., but after repeated injection became cyanotic in appearance.

<div align="center">DISCUSSION</div>

It is of considerable interest that methyl phosphate appears to be utilized in the formation of creatine from guanidoacetic acid by homogenates or extracts of liver tissue of rats.   The formation of creatine appears to be dependent upon the integrity of the methyl phosphate, for formation is reduced when the material is undergoing enzymatic hydrolysis and when oxygen is available for its oxidation by the tissues.

The failure of methyl phosphate to substitute for choline in the growth of white rats receiving homocystine as their source of sulfur can be considered as almost incontrovertible evidence that the methyl phosphate is not utilized normally in transmethylation processes.   It is not the intention of the authors to dismiss methyl phosphate merely because of the toxicity when it is injected into the intact animal, but until the details of the transmethylation are known, it is difficult to consider the results with methyl phosphate as more than an artifact.

<div align="center">SUMMARY</div>

Methyl phosphate has been found to be active in the formation of creatine from guanidoacetic acid by liver tissue of rats.   Methyl phosphate will not substitute for choline in the growth of white rats receiving homocystine as the sulfur of their diet.

<div align="center">BIBLIOGRAPHY</div>

1. Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **134,** 635 (1940).
2. Armstrong, M. D., and Binkley, F., *J. Biol. Chem.*, **174,** 889 (1948).

PREPARATION OF TRIPHOSPHOPYRIDINE NUCLEOTIDE*

By G. A. LePage and GERALD C. MUELLER†

(*From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison*)

The report of Warburg *et al.* (1) on the isolation of triphosphopyridine nucleotide (coenzyme II, TPN) from blood cells is, so far as we know, the only published method for preparing this coenzyme. One of the laboratories most actively engaged in work with TPN enzymes is using this method essentially unchanged, except that their source material is liver rather than blood cells.[1] While this procedure results in preparations of high purity, it is relatively complex and difficult and the yields are low, because of the frequency of steps with 60 to 70 per cent recoveries. In several trials of this method[1] we were able to confirm the results reported.

Some earlier work on the preparation of diphosphopyridine nucleotide (DPN) (2) demonstrated that charcoal adsorption was useful as a purification step for this nucleotide. Through tests on charcoal adsorptions of TPN, a simplified method for the preparation of pure TPN has been developed. In this method, the TPN in heat-inactivated water extracts of pork liver is adsorbed on charcoal and eluted with a water solution of pyridine, the crude TPN being chromatographed on charcoal. Finally, pure material is obtained through the solution of the TPN in acid-methanol and precipitation with ethyl acetate, a step from the method of Warburg *et al.* (1).

EXPERIMENTAL

Facilities Required—In addition to the usual facilities of a chemical laboratory a power-driven meat grinder is needed. A high capacity (12 pounds per minute) model manufactured by the Toledo Scale Company was used in this study and also a pressure filter manufactured by the Sparkler Filter Company, though the latter is not essential and can be replaced by use of Büchner funnels and water-operated suction pumps. If the isolation is to be carried out on a large scale, large sized vessels are required. Cheap, galvanized steel ash-cans of 15 to 30 gallon capacity are very satisfactory.

Assay—In this study it was necessary to have an assay method specific for TPN. This was accomplished by use of a preparation of glucose-6-phosphate dehydrogenase (Warburg's *Zwischenferment*). The enzyme was prepared by a method essentially the same as that of Warburg and Christian (3). A slurry of bottom yeast, obtained from a brewery, was washed three times by suspension in distilled water and filtration each time in a pressure filter. It was then broken up and spread in a thin layer in a fan-equipped drier and was air-dried at 30–35° for 48 hours.

400 gm. of this dried yeast were suspended in 1200 ml. of distilled water and allowed to autolyze for 10 hours at 37°. The suspension was then centrifuged, and about 300 ml. of supernatant were obtained. Of this a 150 ml. batch was diluted to 1500 ml. with distilled water and chilled to 0°. The solution was saturated with CO_2 by bubbling the gas through it for 30 minutes. The resulting sediment was removed by centrifugation at 0° and discarded. The supernatant fluid was diluted to 15,000 ml. with CO_2-saturated distilled water at 30°. This solution was allowed to stand at 30° for 30 minutes, then left at 0° overnight. A precipitate formed, the supernatant fluid was decanted and discarded, and the precipitate centrifuged. This precipitate contained the desired dehydrogenase. It was dried *in vacuo* over P_2O_5. The yield was 1.0 to 1.5 gm. Specificity was demonstrated by incubation with 1.0 mg. of an 80 per cent DPN preparation made by the method of LePage (2). No absorption was obtained at 340 mμ. Glucose-6-phosphate was prepared from starch by use of a rabbit muscle preparation, according to the procedure of Fantl and Anderson (4), modified to include a 5 hour hydrolysis of the sugar phosphates at 100° in 1 N HCl. The glucose-6-phosphate was precipitated as the barium salt with alcohol, decolorized with charcoal, and reprecipitated with alcohol.

Assays for total TPN and DPN were carried out by the hydrosulfite reduction method described by LePage (2) for DPN. When pure TPN preparations were obtained, hydrosulfite assays of these agreed with results obtained by using the glucose-6-phosphate dehydrogenase. Measurements of DPN, when present, were obtained by difference.

For use, 60 mg. of the enzyme preparation were homogenized in 10 ml. of 0.1 per cent $NaHCO_3$ and left at room temperature 20 minutes. The insoluble residue was centrifuged and the resulting slightly opalescent fluid used. This solution retained its activity for at least 1 week when stored at 0°.

TPN concentrates were, if not already in solution, made up in water as 0.15 to 1.0 per cent solutions and 0.1 to 0.3 ml. aliquots were used for each test. The sample was mixed with 0.4 ml. of 0.045 M glucose-6-phosphate, 0.5 ml. of fresh 1 per cent $NaHCO_3$, 0.10 ml. of enzyme solution,

and water to make 3.0 ml. This was mixed and incubated at room temperature 10 minutes (equilibrium was reached in approximately 5 minutes for 200 γ of TPN or less); then absorption was read at 340 mμ in a Beckman quartz spectrophotometer against a blank containing all reagents except the glucose-6-phosphate. A separate correction (very small) could be made for absorption caused by the latter. The TPN content of the sample was calculated with the constant of Warburg (5) ($E = 5.65 \times 10^6$ sq. cm. per mole). In our experimental conditions we multiplied optical density by 396 to obtain micrograms of TPN per sample.

When batches of pork liver of 5 to 50 kilos were run in this study, the same yields and purity were obtained regardless of batch size. The process is described for an exemplary batch. This adheres to the findings as to yield and purity at the various stages obtained in a large number of separate experiments, several of which were run with the final and complete procedure.

Isolation Procedure —A 15 kilo batch of pork liver (eight livers) was obtained directly from the packing-house killing floor as soon after slaughter as available (approximately 20 minutes) and packed in chopped ice. It was transported to the laboratory as soon as possible (30 minutes). The meat grinder was set up over a 15 gallon can containing 15 liters of water at 90–100° with steam flowing into the water rapidly from a supply line (30 pounds pressure). The liver was weighed and run through the grinder directly into the hot water as rapidly as possible (5 to 7 minutes) with stirring of the water during the addition. When all the liver had been added (temperature now 80–90°), heating of the suspension was continued until the pink color, evident when a little of the suspension was dipped up in a beaker, disappeared and for 5 minutes thereafter. Then chopped ice and cold water were added to the vessel with stirring to make a final volume of approximately 60 liters, with the temperature at 25–35°. The suspension was filtered through cheese-cloth and the solid matter pressed as free of liquid as possible and discarded. An ordinary mop-squeezer was found to be satisfactory as a press. A clear filtrate free of suspended meat and fat particles was obtained by filtration of the extract with the addition of approximately 5 gm. of Hyflo Super-Cel[2] per liter. The filtrate now had a volume of 50 liters and was at pH 6.5. Without any further treatment, it was put into a 15 gallon can and 225 gm. of Nuchar C190 decolorizing charcoal[3] were added. This was vigorously stirred with a mechanical stirrer for 15 minutes, then set aside at room

[2] A filter aid supplied by Johns-Manville.

[3] Obtained from the Industrial Chemical Sales Division, West Virginia Pulp and Paper Company, Tyrone, Pennsylvania. It is necessary to specify the "unground" material in ordering the Nuchar C.

temperature for 2 hours to settle, after which time the supernatant fluid was decanted and discarded, leaving 95 per cent of the charcoal as a layer in the bottom of the can. The charcoal was transferred to a Büchner funnel and washed quickly with distilled water.

The charcoal cake was suspended in 2250 ml. of a solution of 10 per cent pyridine in water (by volume) and stirred mechanically for 20 minutes, then filtered with suction on a Büchner funnel. The charcoal was then discarded. The eluate was extracted with two successive 500 ml. portions of chloroform (technical grade, satisfactory). The chloroform layer was discarded each time. This procedure removed most of the pyridine, leaving the solution at approximately pH 6.3. After adjusting the pH to 2.0 with concentrated nitric acid, the water solution was mixed with 4 volumes of cold acetone and left at 0° overnight (or centrifuged immediately). In the morning the acetone solution was poured off and discarded. The crude nucleotides were caked on the sides and bottom of the vessel, and can be washed with acetone and dried if not to be used immediately. When dried, they contain approximately 10 per cent TPN and 20 per cent DPN. If the work is to be carried out immediately, the nucleotides are redissolved in a minimum of water (approximately 150 ml.), filtered at 0°, and assayed for TPN. The insoluble material is discarded. The solution is made to 3 per cent with pyridine and is then pH 5 to 6 and ready to be chromatographed. This solution shows no decline in TPN content at least for several days at 0°.

The columns for the chromatograms were constructed by attaching Pyrex glass tubes of the same diameter to fritted glass Büchner funnels of medium porosity. A large number of chromatograms were run with two sizes of these, one 21 mm., the other 46 mm. inside diameter, and the one with 5 times the area of the other. Since the results with these two sizes were identical as long as flow rates and depth of columns were the same, it seems likely that any size can be used. The procedure will be described for a 46 mm. column.

A granular charcoal known as Nuchar C[3] was chosen for the chromatography. The coarse material was removed by discarding matter that would not pass through a 40 mesh standard sieve. The material used is essentially all retained on a 200 mesh sieve, and the sieved material was acid-washed[4] and dried before use.

---

[4] 150 gm. of Nuchar C, which had been sieved to obtain the material which would pass through 40 mesh, were suspended in 2 liters of 10 per cent hydrochloric acid and heated to boiling, then filtered dry on a Büchner funnel with suction. The use of metal equipment such as metal spatulas was scrupulously avoided. The charcoal cake was resuspended in 2 liters of distilled water, filtered, and the process repeated. Then the cake was dried for 8 to 10 hours in an oven at 85–95°.

Nuchar C was poured into the column dry and tamped down gently with a piece of filter paper on top until a 10 inch depth was obtained. This requires approximately 100 gm. of the charcoal. A light vacuum was applied at the outlet and water added to the column to displace the air. A solution of the nucleotides in 3 per cent pyridine (100 to 150 ml.) containing approximately 5 gm. of the crude material (500 mg. of TPN) was poured onto the column. When this had all just entered the charcoal, a solution of 3 per cent pyridine in water was added, and addition of this continued as long as the column was in operation. A head of approximately 3 inches of solution above the charcoal was sufficient to maintain the desired rate of flow (5 to 10 ml. per minute). The fluid which emerged from the column at first was colorless and below pH 3.0, as indicated by

TABLE I

Results of Charcoal Chromatograph to Purify TPN

A solution of 3 per cent pyridine was the eluant. 500 mg. of TPN were run onto the column in a solution also containing 1000 mg. of DPN.

Fraction No.	Volume	TPN	DPN	Remarks
	ml.	*mg.*	*mg.*	
1	500	0	0	Colorless pH 3
2	50	1	0	" " 4.0
3	50	125	0	Slight color pH 6.2
4	50	160	0	" " " 6.2
5	50	130	0	" " " 6.2
6	50	34	0	" " " 6.2
7	50	10	0	Colorless pH 6.2
8	50	8	5	" " 6.2
9	100	12	300	" " 6.2
Total....	850	480	305*	

* The remaining DPN could be obtained by continuing the elution.

spot plate tests with bromocresol green indicator.[4] The pH was tested every few minutes until it suddenly became pH 4.0 or over. The liquid collected up to this point was termed Fraction 1. Then collection of the fluid was started for Fraction 2 and cuts made every 50 ml. No TPN emerged until this pH change occurred, which was usually after approximately 500 ml. of fluid had filtered through the column. The TPN was present largely in the first four or five cuts after Fraction 1. This is illustrated in Table I. It will be noted that a 96 per cent recovery is obtained from the chromatogram and 90 per cent of the TPN is in Fractions 3 to 6 inclusive, which contain no DPN.

Fractions 3 to 6 inclusive were extracted twice with 50 ml. of chloroform (U. S. P.), treated with 4 ml. of concentrated nitric acid, and pre-

cipitated with 5 volumes of acetone at 0°. The suspension was centrifuged in the cold, washed with acetone, and dried *in vacuo* over sulfuric acid. The material obtained was a white preparation weighing 0.435 gm. and assaying 87 per cent TPN. No DPN was present. Recovery was therefore 76 per cent of the 500 mg. used. An additional 6 per cent present in Fractions 7 to 9 can be added to later columns.

The following operation was carried out with cold reagents, entirely in a room at 0°. A 400 mg. aliquot of the above preparation (ground in a mortar to a fine powder) was suspended for 15 minutes, with mechanical stirring, in 250 ml. of anhydrous methanol containing anhydrous hydrochloric acid (0.1 N). The suspension was filtered through a fritted glass funnel with suction and the filtrate precipitated with 3 volumes of anhydrous ethyl acetate. The flocculent precipitate was centrifuged and dried *in vacuo*, and the supernatant discarded. The precipitate, when dry, was 325 mg. of white material which assayed 107 per cent TPN (by using Warburg constants (1)). The recovery here was 100 per cent. When this preparation was put in a vacuum oven for several hours under high vacuum at 60°, it lost approximately 4 per cent of its weight. However, it is apparently unable to withstand this treatment and the assay dropped to 85 per cent.

Factors Affecting Yields and Recoveries—The most critical factor affecting the yield of TPN is the handling of the liver tissue. It is desirable for the liver to be chilled as soon as possible after the animals are slaughtered. Once the liver is chilled and intact, the coenzyme content is well stabilized. However, in experiments in which the liver was not ground directly into the hot water, yields were reduced 80 to 90 per cent. Destruction of the TPN is apparently very rapid in the ground tissue.

On several occasions, when the heating period was evidently not sufficiently prolonged, a pink color remained in the liver filtrate. Very little TPN was obtained in these cases. Evidently the TPN was not split off the protein, or was destroyed by enzymes not inactivated in the heat treatment.

When the charcoal was filtered from the suspensions, a period of several hours was required for this operation. The added 5 per cent TPN recovered, over that obtained as described, was considered too small an amount to justify this effort.

Tests were made to determine how much charcoal was required to adsorb all the TPN from the liver extracts and to assure that none remained in the filtrate. For this, two approaches were used. Aliquots of the filtrate were precipitated with mercuric acetate as in the procedure of Warburg *et al.* (1), and the mercury salts decomposed with hydrogen sulfide and tested for TPN. None was found when the specified amount

of charcoal had been used. A better check was obtained by use of a second charcoal adsorption and assay of eluates of this adsorption for TPN. It was established in separate experiments that added TPN could be completely recovered from the extracts by charcoal adsorption.

The solubility of TPN in the acetone supernatant was tested by using a charcoal adsorption and testing the eluate, after it had been demonstrated that charcoal would adsorb TPN from 80 per cent acetone. No TPN was recovered, though some DPN was found present in these solutions.

A number of charcoals were studied in order to choose the most suitable one for the initial charcoal adsorption from the liver extracts. This study was carried out by stirring 2 liter aliquots of a liver extract with 10 gm. quantities of the charcoals for 15 minutes, filtering, and eluting each charcoal with 100 ml. of pyridine-water, extracting the pyridine from

TABLE II

Tests of Ability of Various Charcoals to Adsorb TPN from Aliquots of Liver Extract

Charcoal sample	TPN in eluate	Eluate
	mg.	
Norit A...... .	24.0	Clear, highly colored; charcoal settled well
Nuchar C... .. .	29.8	" " " " mainly floated on surface
" C190	31.6	Low in color but opalescent; charcoal settled well
" C115 . ..	18.0	" " " clear; charcoal settled fairly well
" W	31.6	" " " " " " well
" WA.. .	8.7	" " " " " " fairly well

each with chloroform, and assaying enzymatically for TPN. The charcoals were evaluated in terms of three properties, clarity and color of eluate (an indication of purity), capacity to adsorb TPN, and ability to settle after suspension in the extract. On this basis, either Nuchar C190 or Nuchar W would be suitable. The results are shown in Table II.

With Nuchar C190, a brief study was made of the effect of variations in the concentration of pyridine used for elution. Three concentrations were tried, 5, 10, and 20 per cent pyridine (by volume) in pilot batches. The eluates from these three tests contained 7.7, 10.3, and 7.2 mg. of TPN respectively. On precipitation and drying *in vacuo* the preparations were 6.0, 8.0, and 5.9 per cent TPN respectively. The obvious choice was made to use 10 per cent pyridine.

The same six charcoals were studied as to suitability for use in chromatograms, with the exception of Nuchar WA. It was necessary to use

approximately 60 per cent as much Hyflo Super-Cel by weight as charcoal in all cases, except that of Nuchar C, in the columns, as a mixture and suction had to be applied to the receiver to obtain adequate flow rates in these cases. For all these charcoals,[3] the same distribution of TPN and DPN was found in the eluates. However, recoveries were low (34 per cent) in the case of norit A and were only 72 to 85 per cent with the others. When they were given the acid washing and drying treatment,[4] all gave increased recoveries (90 to 96 per cent), and a colored impurity which came through with the TPN from neutral charcoals was completely retained.

One lot of norit A gave low recoveries and this was not remedied by acid washing. One batch of TPN concentrate which had been subjected to a mercury precipitation, and the mercury removed as sulfide, gave low recoveries on chromatograms. Traces of metals are therefore to be avoided.

On one occasion, a liver extract was treated with charcoal and left overnight at 0° to settle, rather than the usual 2 hours. The eluate from this charcoal was highly colored and much difficulty was subsequently encountered in the purification of the TPN concentrate obtained.

The TPN could be assayed by the enzymatic method with either the fractions obtained directly from the chromatographs, in which case 3 per cent pyridine was present, or the solutions from which some of the pyridine had been extracted with chloroform, since neither the pyridine nor the chloroform affected the enzyme appreciably. No TPN is lost in the chloroform-pyridine layers which are discarded, as shown by identical assays obtained with solutions before and after extraction.

Studies were made of the yield of TPN obtained from beef and pork liver processed in identical manner. The yield in terms of TPN found present in the charcoal eluates was 42 mg. of TPN per kilo for pork liver and 32 mg. per kilo for beef liver. As a rule, pork liver is cheaper and more readily available.

When the chromatographs are run with 3 per cent pyridine, the DPN remains on the column until the TPN is almost all eluted. However, the DPN can be completely recovered if more 3 per cent pyridine is run through the column. We chose to ignore the DPN here, since it is much more easily obtained from yeast (2). However, on one occasion the DPN was precipitated and dried, without any attempt at fractionation, and a white preparation obtained which assayed 70 per cent DPN.

Some experiments conducted with yeast which had been heat-inactivated and filtered showed that the direct application of charcoal adsorption to the filtrates was equally successful with yeast. For example the filtrate (100 liters) from 200 pounds of yeast was treated with charcoal, as de-

scribed above for liver, and the charcoal eluted with pyridine. The eluates were extracted and the water layer precipitated with acetone. Material was obtained which assayed 45 per cent TPN-DPN. It contained 8 gm. of DPN and 0.6 gm. of TPN. When this concentrate was extracted with 0.5 N HCl in methanol and precipitated with 3 volumes of ethyl acetate, the TPN was destroyed and the DPN completely recovered. In the final step of our procedure, use was made of a phase of the method of Warburg et al. (1) in which anhydrous methanol containing 0.1 N HCl is used. The latter report a TPN recovery of 95 per cent. We found tsat recoveries were complete (100 per cent) if the whole operation was chrried out rapidly in a refrigerator (0°). However, even if only the fiatration was carried out at room temperature, recoveries dropped to 816 per cent.

DISCUSSION

The procedure described here represents a considerable simplification compared with the methods previously available. The reagents required (charcoal, acetone, pyridine) are relatively cheap and the over-all yield of the TPN in the initial extracts is approximately 80 per cent when final purity is reached. The yields obtainable with the procedure formerly available were in the vicinity of 26 per cent.

TPN made available by this preparative procedure has already made possible the discovery of a TPN-specific enzyme system involved in the destruction of carcinogenic azo dyes by rat liver.[5] It should make possible more wide-spread research on TPN-linked enzymes.

The assays for TPN have all been computed with the constant used by Warburg (5). However, some calculations made recently by Horecker and Kornberg (6) indicate that pure TPN should have an extinction coefficient approximately 11 per cent higher than that of Warburg. Our findings would seem to substantiate these calculations. It would appear that this is the first report in which pure TPN has actually been obtained.

SUMMARY

A procedure is described for preparation of pure triphosphopyridine nucleotide (TPN) from pork liver. This is accomplished by inactivation of the liver in hot water, adsorption of the TPN from the water extract with charcoal, elution with pyridine-water, and precipitation of the TPN with acetone. The preparation is further purified by chromatographing on charcoal and by dissolving in acid-methanol and precipitating with ethyl acetate. The recovery of TPN present in the liver extracts is 80

[5] Unpublished data from Dr. Gerald C. Mueller and Dr. J. A. Miller.

per cent.   Approximately 0.500 gm. of pure TPN is obtained from 15 **kilos** of pork liver.

BIBLIOGRAPHY

1. Warburg, O., Christian, W., and Griese, A., *Biochem. Z.*, **282**, 157 (1935).
2. LePage, G. A., *J. Biol. Chem.*, **168**, 623 (1947).
3. Warburg, O., and Christian, W., *Biochem. Z.*, **254**, 438 (1932).
4. Fantl, P., and Anderson, C. M., *Australian J. Exp. Biol. and Med. Sc.*, **19**, 117 (1941).
5. Warburg, O., *Ergeb. Enzymforsch.*, **7**, 210 (1938).
6. Horecker, B. L., and Kornberg, A., *J. Biol. Chem.*, **175**, 385 (1948).

THE PROTEINS OF GREEN LEAVES

III. EVIDENCE OF THE FORMATION OF TOBACCO MOSAIC VIRUS PROTEIN AT THE EXPENSE OF A MAIN PROTEIN COMPONENT IN TOBACCO LEAF CYTOPLASM*

By SAM G. WILDMAN, C. C. CHEO,† AND JAMES BONNER

(From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena)

One of the remarkable properties of a leaf infected with tobacco mosaic virus is the unusually large amount of virus protein which appears as a consequence of the infection. Some weeks after inoculation, 25 per cent or more of the total protein in tobacco plants can be directly isolated as crystalline virus protein (1, 2, 7, 10). This massive synthesis of virus protein is, however, accomplished without a significant change in either the total or the soluble nitrogen content of tobacco plants (8). In fact, virus formation occurs even in detached and darkened leaves (13), conditions under which protein hydrolysis normally predominates (5). Virus multiplication must occur therefore at the expense of preexisting and normal nitrogenous constituents of tobacco leaf protoplasm.

What, then, are the leaf constituents which are utilized in virus synthesis? Recent work on the protein constituents of the leaf (14, 15) makes it possible to attempt to obtain an experimental answer to this question. The cell-free protoplasm of leaves can be divided into three distinctly different types of nitrogenous materials: (1) The soluble, low molecular weight, nitrogenous constituents such as amino acids, amides, etc. (2) The *particulate matter*, consisting of the nucleus, chloroplasts, and any other as yet unknown materials of a similar particulate nature. It is estimated that 90 to 95 per cent of the particulate matter in spinach leaf protoplasm consists of chloroplasts. Because of the large particle sizes involved, the particulate matter is separated readily from other leaf proteins by moderate centrifugal fields. (3) The soluble proteins of the leaf cytoplasm; the *cytoplasmic proteins*. These proteins are not sedimented even by centrifugal fields up to $43,000 \times g$ for 2 hours. It is thus possible simply and quantitatively to extract the protoplasm of leaf tissue and to separate the protoplasm into particulate and soluble cytoplasmic

protein. It should therefore be feasible to determine whether either the particulate matter or the soluble proteins of the cytoplasm bear a relation to the formation of virus protein.

The cytoplasm of spinach leaves, which has been studied in detail (14, 15), consists of two protein constituents, one of which, *Fraction I protein*, makes up approximately 75 per cent of the total cytoplasmic proteins. This protein contains bound purine, pentose, and phosphorus (15) and is hence a nucleoprotein. Purified Fraction I protein is electrophoretically homogeneous and has a molecular weight, as indicated by osmotic measurements, in excess of 200,000.

Frampton and Takahashi (6) have shown by electrophoretic analysis that a new cytoplasmic protein appears in tobacco leaves after the leaves are infected with tobacco mosaic virus. This new protein component appears to be the virus itself. In the present communication, it will be shown that tobacco leaves contain a main protein component in the cytoplasm analogous to that already described from spinach leaves. Quantitative chemical and electrophoretic analyses of the cytoplasmic proteins of healthy and virus-infected leaves strongly suggest that virus protein is synthesized at the direct expense of this normal protein.

Methods and Materials

Plants—Young *Nicotiana tabacum* plants, variety Havana 38 or Turkish Samsun as indicated, were used in these experiments. Seeds were supplied through the courtesy of Dr. E. E. Clayton of the United States Department of Agriculture, Beltsville, Maryland. The plants were individually grown in pots containing washed sterilized sand, and were supplied with Hoagland's complete nutrient solution including micro elements. They were protected from contamination in an insect-free greenhouse with the usual aseptic precautions. In comparative experiments, plants as uniform as possible were chosen.

Virus—Common tobacco mosaic virus 1, obtained through the courtesy of Professor James Johnson of the University of Wisconsin, was used to infect tobacco plants by the carborundum technique.

Fractionation of Leaf Protoplasm—The methods (14) found useful for the fractionation of spinach leaf protoplasm were only slightly modified for use with tobacco leaves. At all times during the fractionation of the leaf protoplasm, an effort was made to keep the temperature as close to 0° as possible and to work rapidly. The following method was used throughout this study both for healthy and virus-infected leaves.

All leaves were clipped from the stems except for the top clusters which were omitted from the analysis, since they had been used to infect some of the plants with virus. The petioles and midribs were removed and

discarded so that essentially only the parenchymatous cells of the leaves were analyzed. The leaves were weighed, stacked on top of each other, and two leaf disks removed from each leaf with a cork borer. This representative aliquot was weighed, frozen, dried by lyophil, again weighed, and used to estimate the total dry matter contained in the original leaf sample.

The remainder of the leaves was rapidly sliced into approximately 1 sq. cm. pieces, and then ground in an Eppenbach colloid mill. The mill was started circulating with 150 ml. of ice-cold potassium maleate buffer, pH 6.9. It is essential to use a solution buffered near the neutral point in order to prevent precipitation of the cytoplasmic proteins. In the absence of buffer, a pH of about 5 is rapidly attained upon grinding tobacco leaves in water, which is sufficient to cause a considerable isoelectric precipitation of the cytoplasmic proteins. Enough leaves were added to the buffer to produce a thick slurry, which was then freed of cell walls and unbroken cells in a basket centrifuge lined with sharkskin filter paper.

The cell-free protoplasmic juice which passed through the paper was returned to the mill and used in grinding more leaves. This process was repeated until all of the leaves were ground and the protoplasmic contents of the cells dispersed into solution. The concentrated juice was finally passed through two thicknesses of sharkskin filter paper to insure complete removal of all cell walls. The final pH of the juice was 6.7. The residues remaining on the paper were dried and weighed. Since the total amount of dry matter was ascertained from the aliquot, it was possible to calculate the total amount of dry matter dispersed as a cell-free juice. In all instances, 60 to 65 per cent of the total dry matter was found in the cell-free juice. Since about 10 per cent of the original dry matter consisted of insoluble cellulose, it is evident that more than 65 per cent of the leaf cells was ruptured by the colloid mill treatment, with consequent extraction of their protoplasmic contents as a cell-free juice.

The filtered and concentrated protoplasmic juice was immediately frozen with the aid of a dry ice-methyl cellosolve bath. This freezing treatment results in aggregation of the particulate matter so that when the suspension is thawed, the particulate matter can be completely sedimented by centrifuging for 1 hour at $5000 \times g$. The sediment was dried and the dry weight taken to represent the total particulate matter of the protoplasm. The clear brown supernatant was distributed into 1 inch Visking casings which were placed before a fan until the contents were reduced to about one-third of their original volume, usually about 25 ml. of concentrated juice per 100 gm. of fresh leaf tissue. The content of total cytoplasmic proteins was about 3 per cent. After tying off the sacs

to prevent excessive dilution, the concentrated protein solution was dialyzed with mechanical stirring for 48 hours against two separate 2 liter portions of buffer. The total amount of cytoplasmic proteins in the sample was ascertained from an aliquot, and the protein after dialysis was rapidly frozen and stored at $-16°$ until it was used for electrophoresis analysis. Freezing produced no apparent deleterious effect on the proteins since, when thawed, the protein solution remained perfectly clear. Protein determinations were made either by drying or by trichloroacetic acid precipitation as described previously (14).
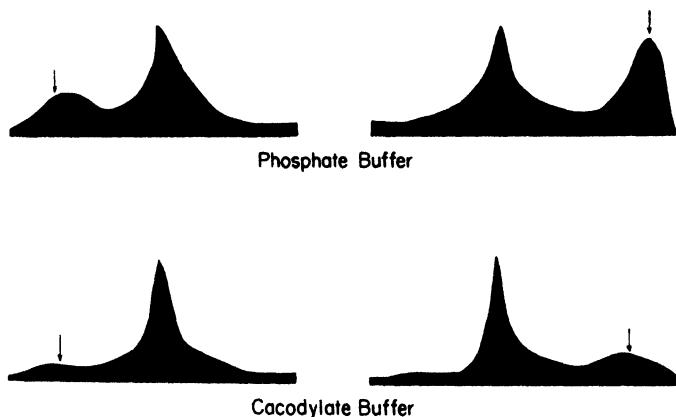
FIG. 1. Comparison of the electrophoretic behavior of Havana tobacco leaf cytoplasmic proteins in phosphate and cacodylate buffer. 1.0 per cent solutions of total proteins in buffer of 0.1 ionic strength, pH 6.9. Migration time, 180 minutes at 15 ma. Arrows indicate positions of starting boundaries. Right, ascending; left, descending.

Electrophoresis—Through the courtesy of Dr. S. Swingle of the Chemistry Department of this Institute, cytoplasmic proteins were examined in a modified Tiselius electrophoresis, moving boundary, apparatus with the Longsworth scanning device (12). Conventional, double length analytical cells were used. Before analysis, the proteins were previously dialyzed against a 0.0233 M cacodylic acid buffer which was brought to pH 6.9 and a total ionic strength of 0.1 by the addition of 0.02 M NaOH and 0.08 M NaCl. This univalent ionic buffer gave much more satisfactory resolution of cytoplasmic protein mixtures than phosphate or a buffer such as potassium maleate. With phosphate buffer for example, anomalous δ boundaries were encountered such as are illustrated by Fig. 1, where it can be observed that a pronounced boundary at the original protein buffer interface is still present even after the cytoplasmic protein mixture

had been allowed to migrate for 3 hours. However, in the presence of cacodylate at the same pH and ionic strength as phosphate, a normal spreading of the starting boundary was found after the protein mixture had migrated for 3 hours. Cacodylate buffer was therefore used in all subsequent electrophoresis experiments.

In comparing the cytoplasmic proteins obtained from healthy and virus-infected leaves, analysis was performed in paired electrophoresis cells at identical total protein concentrations. Both samples therefore received the same treatment as far as current, migration time, etc., were concerned. The proteins were usually allowed to migrate for 2 or 3 hours with a current of 15 ma. At pH 6.9, this time of migration was adequate for the separation of virus protein from the normal protein of tobacco leaf cytoplasm.

<div align="center">EXPERIMENTAL</div>

Electrophoretic Analysis of Cytoplasmic Proteins of Healthy Tobacco Leaves—Cytoplasmic proteins were prepared, as described above, from two varieties of *Nicotiana tabacum*, Havana 38 and Turkish Samsun, and from *Nicotiana glutinosa*. After removal of the particulate matter, the cytoplasmic proteins were examined in a Tiselius electrophoresis apparatus, and tracings made from the scanning patterns are presented in Fig. 2. The three preparations are strikingly similar in that each appears to contain a protein component comprising 75 per cent or more of the total cytoplasmic proteins. Even after 3 hours migration at 15 ma. in cacodylate buffer, pH 6.9, there is no evidence of electrophoretical inhomogeneity in the principal component in any preparation. The similarity of these cytoplasmic protein spectra to spinach cytoplasm is further augmented by the presence of minor protein components which, in the case of Havana and Turkish tobacco, appear as faster moving components than the main protein, while with *N. glutinosa*, the minor component moves more slowly. Therefore, it can be concluded that tobacco leaves are closely similar to spinach leaves as far as the general protein spectrum of the normal cytoplasm is concerned.

An indication of the experimental error involved in this type of quantitative protein analysis is shown by the data in Table I. Two sets of fourteen Havana tobacco plants were separately analyzed for total particulate proteins and total cytoplasmic proteins by the methods described above. The data show that, on a dry weight basis, the amount of particulate protein isolated from the two sets of leaves agreed within 6 per cent, while the amounts of total cytoplasmic proteins agreed within 5 per cent. In order to exaggerate any differences in the ratio of total particulate proteins to total cytoplasmic proteins which might result from

insufficient grinding of the leaf cells, the second sample was ground to a lesser extent in the colloid mill than the first. However, on a dry weight basis, the yield of the two constituents was essentially the same and the

N glutinosa   Havana

Turkish
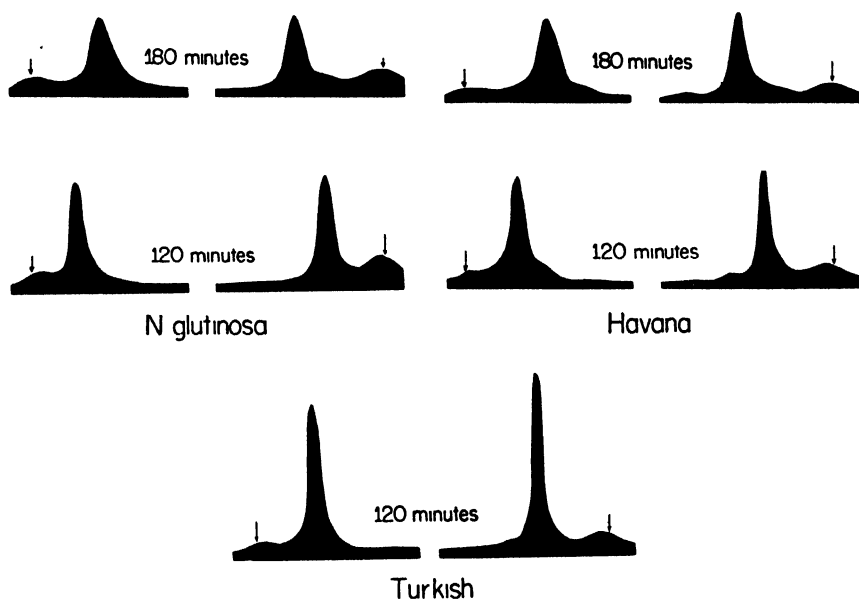
Fig. 2. Electrophoretic patterns of the cytoplasmic proteins of different tobacco leaves. Cacodylate buffer of 0.1 ionic strength, pH 6.9. Current, 15 ma. 1.0 per cent solutions of Havana tobacco and *N. glutinosa* proteins; 1.3 per cent solutions of Turkish tobacco proteins. Right, ascending; left, descending.

TABLE I

Duplicate Analysis of Total Particulate Proteins and Total Cytoplasmic Proteins of Havana Tobacco Leaf Protoplasm

Leaves analyzed in March.

Sample No.	Fresh weight analyzed	Per cent dry weight	Per cent dry weight extracted	Mg. per gm. dry weight of leaves	
				Total particulate proteins	Total cytoplasmic proteins
	gm.				
1	323.3	10.05	63.4	148	84
2	283.3	10.02	54.5	158	80

ratios were unchanged. Evidently, these methods can be used here quantitatively to detect changes in either the particulate or the cytoplasmic constituents of the leaf as a result of virus multiplication.

Electrophoretical Character of Cytoplasmic Proteins Obtained from Healthy and Virus-Infected Tobacco Leaves—In December, 1948, two sets of thirty-three Havana tobacco plants were chosen for uniformity and arranged for experimental use. One set of plants was inoculated with tobacco mosaic virus; the other set was kept as a virus-free control. The top two leaves only of each plant were inoculated with a crude preparation of virus by means of the carborundum technique. The plants were approximately 1.5 months old, averaged about 12 inches in height, and had eight to ten well expanded leaves. 13 days after inoculation, all but the top cluster of leaves from each set of plants were removed and analyzed for particulate protein and soluble cytoplasmic protein. The only evidence of virus infection in the inoculated leaves at this time was the appearance

TABLE II

Effect of Tobacco Mosaic Virus Infection on Total Protein Composition of Havana 38 Tobacco Leaves

December, 1948; leaves analyzed 13 days after inoculation.

Condition of leaves	Fresh weight analyzed	Dry weight	Dry weight extracted as cell-free juice	Mg. per gm. dry weight of leaves		Amount of virus protein in cytoplasm
				Total particulate proteins	Total cytoplasmic proteins	
	gm.	*gm.*	*per cent*			*per cent*
Normal	477	31.3	64.8	160	109	0
Virus-infected	463	35.5	64.5	150	113	37

of a slight amount of vein clearing in the leaves which had been used for inoculation, but which were not included in the protein analysis. Other than these very mild symptoms, the control and infected leaves gave every appearance of being alike.

The results of the protein analyses are given in Table II together with the electrophoretic scanning patterns of Fig. 3. From Table II it is evident that the weights of the two lots of leaves were closely similar and, more important, that the same proportion of dry matter, 65 per cent, was dispersed as a cell-free protoplasmic juice. Similarly, the amount of particulate matter contained in the leaf protoplasm of virus-infected leaves was closely similar to the amount contained in the healthy leaves. The total amount of cytoplasmic proteins in the two instances was also nearly the same. Therefore, it can be concluded that there had been no great change in either the total amount of protein of the leaf protoplasm or the distribution of particulate protein compared to the cytoplasmic protein as the result of 13 days infection with virus.

However, electrophoretic analysis of the soluble cytoplasmic protein contained in the healthy and virus-infected leaves showed that a profound

change had occurred in the physical properties of the proteins as a result of infection.   As shown in Fig. 3, a new and distinctly different protein had appeared.   Since the concentration of total protein used for electrophoresis was in both instances the same, it is possible to compare amounts by planimeter measurements (4), and this comparison shows that 37 per cent of the total cytoplasmic protein of the virus-infected leaf consists of the new component.   Correspondingly, the virus-infected leaf pos-
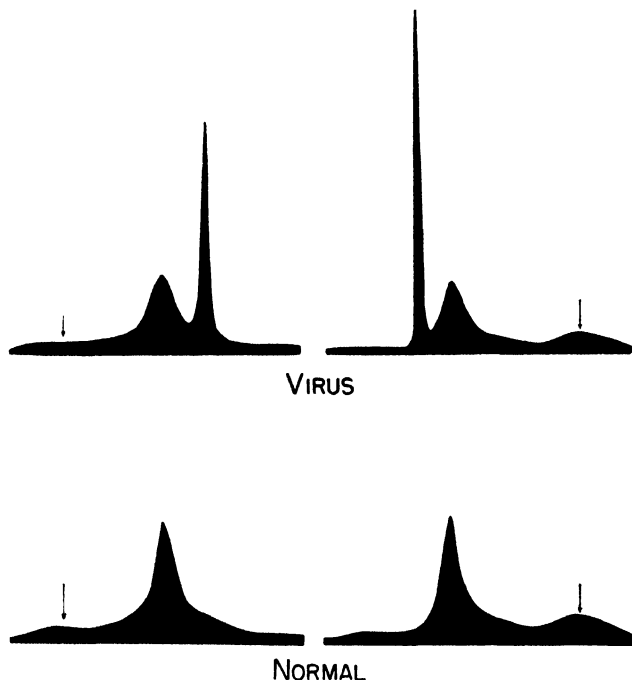
FIG. 3. Comparison of the electrophoretic behavior of the total cytoplasmic proteins obtained from normal and virus-infected Havana tobacco leaves.   1.0 per cent solutions of total proteins.   Conditions the same as in Fig. 2.   Migration time, 180 minutes.   Right, ascending; left, descending.

sessed 37 per cent less normal protein than the non-infected leaf.   It can also be observed that the mobility of the normal protein in both kinds of leaves was the same, but that the new component in the virus-infected leaves migrates more rapidly than the main protein.

Two facts suggest that the new component is indeed virus protein similar in properties to the virus previously isolated in crystalline condition (11).   In the first place, the narrowness of the faster moving peak suggests a protein of very high molecular weight, which consequently diffuses

during the time of electrophoresis at a much slower rate than the normal protein. Secondly, if the protein mixture is allowed to migrate for 6 hours, sufficient separation of the virus protein from the normal protein occurs so that it is possible to remove the faster moving virus component with little or no contamination by the normal protein. When tested on *N. glutinosa*, the new component produced local lesions at high dilutions in a manner characteristic of crystalline tobacco mosaic virus preparations.
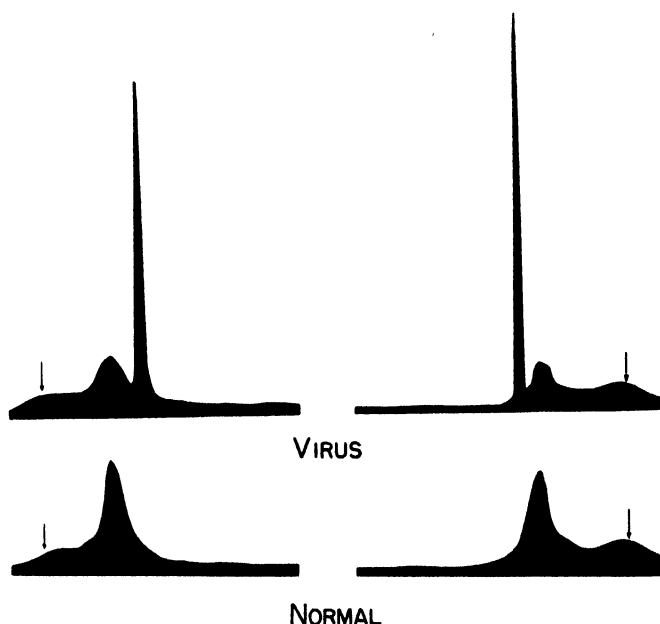
VIRUS

NORMAL

FIG. 4. Comparison of the electrophoretic behavior of the total cytoplasmic proteins obtained from normal and virus-infected Turkish tobacco leaves. 0.8 per cent solutions of total proteins. Migration time, 180 minutes. Other conditions the same as in Fig. 2. Right, ascending; left, descending.

The data in Table II show the total amount of cytoplasmic proteins found in a kilo of fresh leaves. Since in the virus-infected leaves 37 per cent of the total cytoplasmic proteins is the new virus component, calculation reveals that 3.2 gm. of virus protein per kilo of fresh leaves are present 13 days after inoculation. This yield compares favorably with those previously reported by others (3, 7).

Experiments similar to that of Table II and Fig. 3 have been carried out on five separate occasions, and the results in every instance suggest that the appearance of tobacco mosaic virus protein is related to the

disappearance of a corresponding amount of the normal protein of tobacco leaf cytoplasm. For example, the experiment was repeated in January, 1949, with another set of Havana tobacco plants, except that the leaves were harvested 5 days after inoculation. Even with this short time of incubation after inoculation and in complete absence of symptoms suggestive of virus infection, electrophoretic analysis indicated that about 20 per cent of the normal protein had disappeared and a corresponding quantity of the new virus component had appeared. The rapidity of virus protein formation will be brought out shortly in connection with another experiment. When the experiment was repeated with Turkish tobacco leaves, approximately 35 per cent of the main protein component contained in these leaves had disappeared in favor of virus protein 14 days after infection, as shown in Fig. 4.
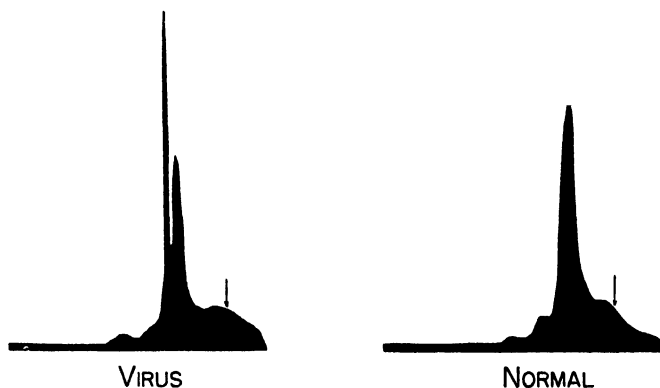
VIRUS   NORMAL

FIG. 5. Comparison of the electrophoretic behavior of the minor protein components of Havana tobacco leaf cytoplasm obtained from normal and virus-infected leaves. 1.5 per cent solutions of total proteins. Migration time, 60 minutes. Other conditions the same as in Fig. 2. Ascending boundaries.

The scanning pattern illustrated by Fig. 5 shows that virus formation does not seem to occur at the expense of the cytoplasmic proteins other than the main protein. 1.5 per cent solutions of normal and virus-infected Havana tobacco cytoplasm were allowed to migrate for 1 hour. At this increased protein concentration and shorter period of migration, some of the proteins comprising Fraction II (14) of cytoplasm can be observed as fast moving components. However, they are present to about the same extent in both samples, even though 37 per cent of the total cytoplasmic protein in the virus-infected leaves represents virus protein. Evidently, there is not only insufficient protein in this fraction to account for the observed synthesis of virus protein, but there is also

no evidence that the quantity of the protein mixture has been significantly decreased as a consequence of virus formation.

Formation of Virus Protein As Function of Time after Inoculation— Turkish tobacco plants, approximately 1.5 months old, were divided into seven groups of fifteen plants each. The experiment was performed in January, 1949. A terminal leaf of each plant was inoculated with a crude
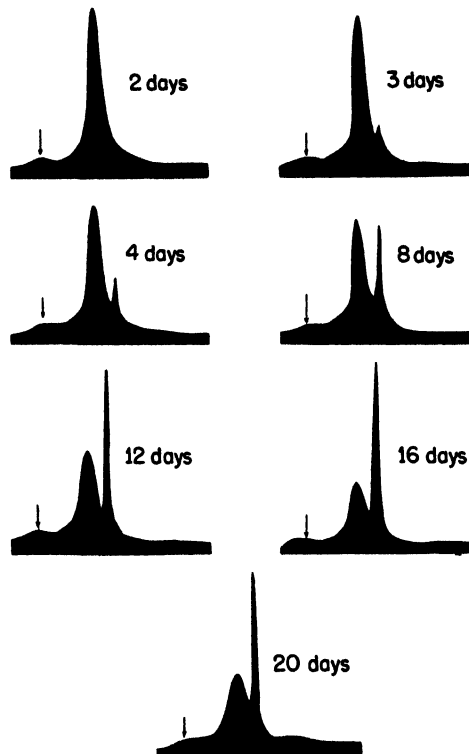
FIG. 6. Time course of virus protein formation in the cytoplasm of Turkish tobacco leaves. 1.0 per cent solutions of total proteins. Migration time, 120 minutes. Other conditions the same as in Fig. 2. Descending boundaries.

preparation of tobacco mosaic virus. With the exception of the top cluster of leaves, all of the lower leaves of fifteen plants were removed 2, 3, 4, 8, 12, 16, and 20 days after inoculation. The soluble cytoplasmic proteins of each sample were isolated and examined for the presence of virus protein by the Tiselius electrophoresis method. For comparative purposes, 1.0 per cent solutions of total cytoplasmic proteins were allowed to migrate for 120 minutes at 15 ma. in cacodylate buffer, pH 6.9. The results of electrophoresis are shown in Fig. 6.

No new component was detected in the leaves removed 48 hours after inoculation, but the unmistakable appearance of a new component in the cytoplasm of leaves removed 3 days after inoculation is evident. There was a progressive increase in the quantity of the new component as the length of time after inoculation increased up to the 12th day, when about 40 per cent of the total cytoplasmic proteins consisted of virus protein. Virus protein formation then appeared to level off and there was no further increase in the amount of the new component when the leaves were analyzed 16 and 20 days after inoculation. Simultaneously with the appearance of the new component, there was an equivalent decrease in the amount of the normal protein of the cytoplasm, and this reciprocal relationship obtains during the entire course of the experiment.

TABLE III

Effect of Tobacco Mosaic Virus Infection on Total Protein Composition of Havana 38 Tobacco Leaves

April, 1949.

Condition of leaves	Days after inoculation	Dry weight of leaves	Dry weight extracted as cell-free juice	Mg per gm. dry weight of leaves		Virus protein in total cytoplasmic protein
				Total particulate proteins	Total cytoplasmic proteins	
		per cent	*per cent*			*per cent*
Normal ..		11.0	64.8	146	87	
Virus-infected ..	5	10.8	64.4	144	80	0
Normal..		10.9	65.7	145	81	
Virus-infected	12	11.4	63.7	136	84	20
Normal. . .		11.2	64.1	161	70	
Virus-infected	17	12.4	64.4	146	78	40

It should be noted again that maximum virus protein formation occurred in leaves which gave no outward signs of virus infection except slight symptoms indicative of virus in the inoculated leaf 12 days after infection, but it will be recalled that this leaf and closely adjacent leaves were not included in the analysis.

The time experiment was repeated in April, 1949, with Havana tobacco plants approximately 12 inches high, having about twelve leaves per plant. Growth at this season of the year was rapid, as evidenced by the rapid expansion of the young leaves compared to the slow growing conditions encountered in the December experiments. As before, a single terminal leaf was inoculated with a crude virus preparation, but this leaf was not included in the subsequent protein analyses.

96 plants were divided into three sets of thirty-two plants each. One-

half of the plants was inoculated with virus; the remainder was kept as virus-free controls. 5, 12, and 17 days after infection, the lower leaves were removed from one set of virus-infected and one set of control plants and the leaves analyzed for total particulate and total cytoplasmic proteins. The cytoplasmic proteins were investigated for the presence of
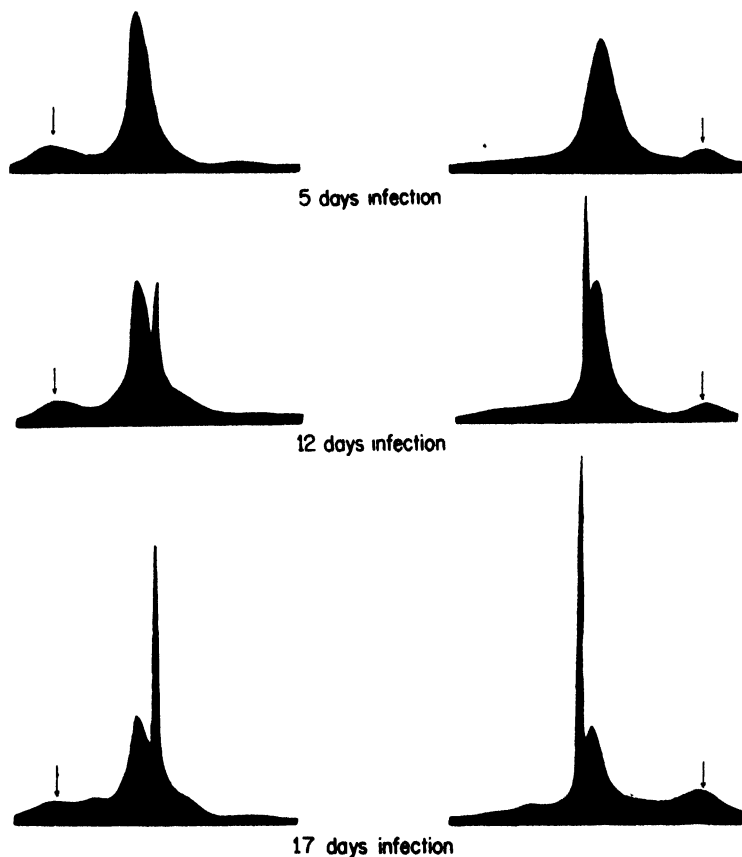
FIG. 7. Time course of virus protein formation in the cytoplasm of Havana tobacco leaves. 1.0 per cent solutions of total proteins. Migration time, 120 minutes. Other conditions the same as in Fig. 2. Right, ascending; left, descending.

virus protein by electrophoretic analysis. No characteristic symptoms of virus infection were detected until 14 days after inoculation. On the 17th day, symptoms were apparent on the younger leaves of all of the infected plants. The results of the protein analysis are presented in Table III and in the scanning pattern tracings in Fig. 7.

Virus protein formation was not as rapid in this experiment as in the previous experiment with Turkish tobacco, but by the 12th day after infection, it is evident that virus protein constitutes about 20 per cent of the total cytoplasmic proteins, while by the 17th day, 40 per cent of the total cytoplasmic proteins consists of virus protein. The data of Table III show that no significant change in total amount of cytoplasmic proteins occurred during the time of this experiment irrespective of whether the leaves were infected with virus or not, even though it is evident from electrophoresis that 12 and 17 days after inoculation the cytoplasm contained substantial amounts of the new virus protein which was absent from the control leaves. There is a small and probably insignificant change in the total particulate proteins as between virus-infected and healthy leaves, but the fact that the virus-infected leaves still contain the same amount of particulate matter on the 17th day after infection as on the 5th day strongly suggests that virus protein formation does not occur at the expense of the chloroplasts or other particulate materials. The data in Tables II and III could be interpreted as showing a slight reduction in particulate matter during virus protein formation. However, the reduction is no greater than that anticipated by the data in Table I, where the amounts of particulate matter from two sets of healthy leaves are compared, but the experimental errors inherent in this type of analysis are still large enough to make a positive statement impossible with the present data.

Evidence has already been presented that the minor protein components of cytoplasm do not change markedly during virus protein synthesis. It seems reasonable to draw the conclusion that virus protein is formed principally at the expense of the main protein component of tobacco cytoplasm.

DISCUSSION

The electrophoresis experiments described above seem to establish two facts concerning the formation of tobacco mosaic virus protein in Turkish and Havana tobacco leaves. (1) Virus protein formation is a process which appears to be completed in the parenchymatous cells of leaves about 2 weeks after inoculation of a terminal leaf. (2) Virus protein appears to be formed at the expense of a normal protein already present in the cytoplasm of tobacco leaves.

The formation of tobacco mosaic virus protein as a function of time after inoculation was investigated by Stanley (10) shortly after he succeeded in isolating the virus in crystalline condition. Virus was isolated from expressed leaf juice at weekly intervals after inoculation, and it was found that the amount of virus protein which could be isolated reached

a maximum about 6 weeks after infection. At first sight, these results seem to be at variance with the conclusions reached above. A proper evaluation of the discrepancy can be achieved, however, by a consideration of the different extraction methods and different tissues used for analysis. In Stanley's experiments, the entire aerial portion of the plant was frozen and passed through a meat grinder, and the juice expressed from the ground pulp by pressure. His data show that less than 25 per cent of the total nitrogen contained in the tissue was actually recovered in the juice in the sample analyzed 1 week after inoculation. Such a low recovery doubtless indicates that many cells were left unruptured by the grinding process. This method is to be contrasted with the grinding procedure used here in which it has been shown that extraction of about 75 per cent of the total leaf nitrogen is achieved (14). Chibnall (5) has shown that intact cell walls act as ultrafilters, and although soluble small molecular constituents can be expressed from tissues whose semipermeability has been destroyed by freezing, etc., the proteins are nevertheless largely retained within the cell. The fact that the first samples of sap analyzed by Stanley were essentially free of trichloroacetic acid-precipitable protein, and were not heavily charged with green color, suggests again that only relatively small amounts of leaf cell protoplasm were extracted as cell-free juice. With the methods used here, the cell-free extract is not only intensely green in color, but it also gives voluminous precipitates when trichloroacetic acid is added to the extract. It is therefore possible that the delayed appearance and relatively slow increase in virus protein in juice expressed in Stanley's experiments may be attributed to incomplete grinding of the leaf tissue. Secondary factors operative in the diseased plants over long periods apparently increase the ease with which the virus may be recovered, although secondary increases in virus content perhaps in tissues other than the parenchyma of the leaves may also be involved.

The fact that formation of virus protein in leaf cells under our conditions seems to reach a maximum about 12 to 14 days after inoculation of apical leaves makes much of the previous work on the mechanism of virus formation of doubtful significance, because most studies have generally utilized plants which were inoculated some 2 to 3 weeks before the investigation (9). The same criticism can be made of experiments in which virus formation is explained on the basis of transformation of the chlorophyll protein (17). Since maximum virus synthesis can be achieved before changes in chlorophyll protein can be detected, it is probable that any subsequent disappearance of chlorophyll is a secondary result of derangement of the cytoplasm by virus. The main protein component in spinach cytoplasm is an enzyme having to do with phosphorus metabo-

lism, and there is evidence that the normal protein of tobacco cytoplasm performs this same function. If a large portion of this protein were replaced by a metabolically inactive protein, *e.g.* virus, unable to fulfil this rôle in the normal cellular metabolism, wide-spread secondary effects on the physiology of the plant might become manifest, such as maintenance of chloroplastic protein level, as well as the ultimate stunting of the infected plants.

Since virus protein appears to be made at the expense of the normal protein in tobacco cytoplasm, the question remains as to how much of a change has occurred when the normal protein is transformed into virus protein. Does the change involve extensive breakdown followed by a new and constitutionally different synthesis of virus protein, or is the transformation process more direct, perhaps involving only a polymerization of the protein? Comparison of the amino acid and nucleic acid contents of the two proteins, the extent of cross reaction between the antibodies formed from the virus and the normal protein,[1] and comparative analysis of the phosphorus relationships of the two proteins (16) should be useful properties in deciding between the alternatives expressed here in their extreme forms.

SUMMARY

Evidence is presented suggesting that tobacco mosaic virus protein is synthesized in Turkish and Havana tobacco leaf cells at the expense of a normal nucleoprotein found in the cytoplasm of the leaf cells. Virus protein can be detected by electrophoretic methods 3 days after inoculation of apical leaves and progressively increases in amount in the lower leaves up to the 12th day after inoculation. The process of virus synthesis appears to reach a static level about 12 days after infection. There is a simultaneous and proportional decrease in the normal nucleoprotein as virus protein is formed.

BIBLIOGRAPHY

1. Bawden, F. C., Plant viruses and virus diseases, Waltham (1943).
2. Bawden, F. C., and Pirie, N. W., *Proc. Roy. Soc. London, Series B*, **123,** 174 (1937).
3. Bawden, F. C., and Pirie, N. W., *Brit. J. Exp. Path.*, **27,** 81 (1946).
4. Bull, H. B., Physical biochemistry, New York (1943).
5. Chibnall, A. C., Protein metabolism in the plant, New Haven (1939).

---

[1] Antibody reagents for both normal protein and virus now under study are found to cross-react with the heterologous antigens. The nature and significance of these cross-reactions are being investigated. Owen, R. D., and Wildman, S. G., unpublished.

6. Frampton, V. L., and Takahashi, W. N., *Arch. Biochem.*, **4**, 249 (1944).
7. Gaw, H. Z., *Arch. ges. Virusforsh.*, **3**, 347 (1947).
8. Martin, L. F., Balls, A. K., and McKinney, H. H., *Science*, **87**, 329 (1938).
9. Spencer, E. L., *Plant Physiol.*, **16**, 663 (1941).
10. Stanley, W. M., *J. Biol. Chem.*, **121**, 205 (1937).
11. Stanley, W. M., *Am. J. Bot.*, **24**, 59 (1937).
12. Swingle, S., *Rev. Sc. Instruments*, **18**, 128 (1947).
13. Takahashi, W. N., *Phytopathology*, **31**, 1117 (1941).
14. Wildman, S. G., and Bonner, J., *Arch. Biochem.*, **14**, 381 (1947).
15. Wildman, S. G., Campbell, J. M., and Bonner, J., *Arch. Biochem.*, in press.
16. Wildman, S. G., Campbell, J. M., and Bonner, J., *J. Biol. Chem.*, **180**, 273 (1949).
17. Woods, M. W., and DuBuy, H. G., *Phytopathology*, **31**, 978 (1941).

METABOLIC FUNCTIONS OF BIOTIN

III. THE SYNTHESIS OF CITRULLINE FROM ORNITHINE IN LIVER TISSUE FROM NORMAL AND BIOTIN-DEFICIENT RATS*

By PATRICIA R. MacLEOD, SANTIAGO GRISOLIA, PHILIP P. COHEN, AND HENRY A. LARDY

(From the Departments of Biochemistry and Physiological Chemistry, University of Wisconsin, Madison)

In Paper II of this series (1), it was shown that the biotin-deficient rat fixes less CO_2 into tissue arginine than does the normal rat. Since the synthesis of urea in the body represents one of the most active CO_2-fixing reactions and since arginine is one of the intermediates of the Krebs-Henseleit (2) cycle, it seemed of interest to investigate the influence of biotin deficiency on this system. While the stage of CO_2 fixation was clearly indicated from the Krebs-Henseleit scheme, the actual demonstration of CO_2 fixation in the synthesis of urea was first reported with the use of isotopic carbon in liver slices (3, 4). A more detailed study of the fixation of CO_2 in the synthesis of urea by cell-free systems confirmed the fact that CO_2 was fixed in the over-all step ornithine → citrulline (5). It therefore was decided to determine the relative activity of the enzyme system catalyzing the synthesis of citrulline from ornithine in the livers of biotin-deficient and normal rats. The effect of pair feeding the controls, the effect of added biotin *in vivo* and *in vitro*, and the effect, *in vitro*, of added sources of the presumed biotin coenzyme were also studied.

EXPERIMENTAL

The animals used in the following experiments were weanling male albino rats of the Sprague-Dawley strain. The casein control ration consisted of the following: alcohol-extracted casein 18, sucrose 72.5, corn oil 5, and Salts IV (6) 4.5 parts. Vitamins were added in the following amounts per 100 gm. of ration: thiamine 30 γ, riboflavin 60 γ, niacin 200 γ, pyridoxine 30 γ, calcium pantothenate 300 γ, *p*-aminobenzoic acid 500 γ, biotin 1 γ, folic acid 2.5 γ, inositol 10 mg., and choline 20 mg. 2 drops of haliver oil were administered weekly. Animals were raised on two deficient rations, one of which contained 18 parts of dried raw egg white substituted for casein (Ration A), and the other 18 parts of casein plus

13 parts of dried raw egg white added at the expense of the sucrose (Ration B). Other constituents of the deficient rations were the same as the casein control ration, with the omission of biotin. Rats fed deficient Ration A generally did not survive beyond 10 weeks, while those fed deficient Ration B survived for much longer periods. The main group of control animals was raised on the casein diet and was pair-fed with biotin-deficient rats.

A second control group, consisting of animals pair-fed with the deficient rats on Ration A, was fed the same egg white-containing ration but was injected intraperitoneally with 50 γ of biotin on alternate days. A third control group received similar injections but was fed the deficient Ration A *ad libitum*. Riboflavin and vitamin B_6-deficient animals were raised on the casein control diet with the omission of riboflavin and pyridoxine, respectively.

For experimentation, the animal was decapitated and the liver quickly removed and chilled. It was homogenized in sufficient cold isotonic KCl to make a 20 per cent suspension. The twice washed residue of the liver homogenate was then prepared as described by Cohen and Hayano (7).

All incubations were carried out in Warburg flasks at 37° for 20 minutes, with air as the gas phase. Two levels of the same washed residue suspension were tested. These were 0.3 ml. and 0.5 ml., generally containing 5 to 6 mg. of nitrogen per ml. The final substrate concentrations were L-ornithine 0.0025 M, L-glutamic acid 0.038 M, $NaHCO_3$ 0.005 M, NH_4Cl 0.005 M, $MgSO_4$ 0.004 M, adenosine triphosphate 0.002 M, phosphate buffer, at pH 7.15, 0.012 M, and KCl to bring the medium to isotonicity. The total volume was 4.0 ml. After 20 minutes incubation, 10 ml. of 1 N HCl were added to stop the reaction. The contents of the flasks were heated 3 minutes in a boiling water bath, and the insoluble protein filtered off. Citrulline was determined colorimetrically on the filtrate according to the method of Archibald (8), as employed by Cohen and Hayano (9). The results are expressed as micromoles of citrulline formed per mg. of washed residue nitrogen.

A *heated extract* was prepared from both biotin-deficient and casein control rat livers, according to a method previously described (10).

Heated residue was also prepared from both biotin-deficient and casein control animals. The washed residue of liver homogenate was prepared in the usual manner and the final suspension of 20 per cent residue in KCl was heated 10 minutes in a vigorously boiling water bath. After cooling it was rehomogenized and stored at 0° until used.

Results

The effect of a biotin deficiency upon the synthesis of citrulline by washed residue of rat liver homogenate is shown in Table I. There was no signifi-

TABLE I

Effect of Biotin Deficiency upon Citrulline Synthesis by Rat Liver Homogenates

Ration fed	Days on experiment	Weight of rat	Citrulline per mg. N*	Mean and standard error
		gm.	*μM*	
Cascin pair-fed control	15	77	1.22	1.27 ± 0.14
	29	120	1.09	
	32	162	0.40	
	35	135	1.29	
	45	172	1.47	
	53	204	1.70	
	57	181	0.76	
	58	193	1.83	
	65	146	2.25	
	66	194	0.42	
	71	212	1.26	
	175	205	1.45	
	190	225	1.35	
Biotin-deficient Ration A† + injected biotin, pair-fed	61	224	1.26	1.28 ± 0.09
	69	217	1.15	
	71	199	1.45	
Biotin-deficient Ration A	19	60	0.81	0.48 ± 0.07
	24	60	0.36	
	30	59	0.65	
	32	115	0.45	
	45	157	0.90	
	48	92	0.38	
	53	159	0.21	
	57	114	0.42	
	58	149	0.59	
	59	134	0.15	
	64	90	0.34	
	71	126	0.45	
Biotin-deficient Ration B	29	127	0.65	0.66 ± 0.05
	35	151	0.89	
	65	151	0.53	
	169	204	0.39	
	171	194	0.72	
	190	247	0.56	
Natural stock ration *ad libitum*		200—400	1.23	1.43 ± 0.23
			2.24	
			0.88	
			1.72	
			0.78	
			2.18	
			1.03	

* Each value represents the average of two experiments.

† These rats were last injected 3 hours before being used.

cant difference in rate of citrulline synthesis between animals pair-fed the casein control diet and those pair-fed the deficient diet with intraperitoneal injection of 50 γ of biotin on alternate days. Further, the rates of synthesis in these two groups did not differ significantly from the rate in those animals receiving a natural stock diet *ad libitum*. However, the rate of citrulline synthesis by washed liver residue from both biotin-deficient groups was decreased about 50 per cent below that of the controls. Since the individual values in all the groups varied over a considerable range, the data of the two control groups and the two deficient groups were pooled in order that the difference between the means of the two groups and the
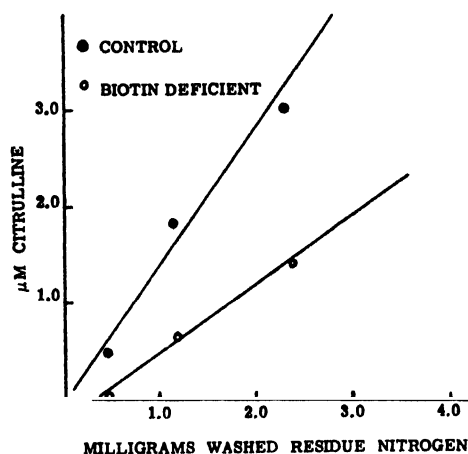
FIG. 1. Influence of tissue concentration upon citrulline synthesis by the washed residue of normal and biotin-deficient rat liver homogenates.

standard error of this difference could be calculated. The difference between the groups is 0.74 ± 0.16, and since this difference exceeds 3 times its standard error, it is statistically significant.[1]

The relationship between the rate of citrulline synthesis and tissue concentration is shown in Fig. 1. Within the range of tissue concentration studied a linear relationship is apparent. However, in the case of the washed residue from the biotin-deficient rat, it is to be noted that not only

---

[1] These were calculated as follows:

$$\text{Standard deviation, } \sigma = \pm_{/}$$

$$\text{Standard error of the mean, } \epsilon(M) \quad \pm \overline{\sqrt{N}}$$

$$\text{Standard error of difference between two means, } \epsilon(D) = \pm\sqrt{\epsilon(M_1)^2 + \epsilon(M_2)^2}$$

is the slope smaller but also the curve does not pass through the origin, suggesting a dilution effect.

Table II shows the effect of the injection of biotin into biotin-deficient rats. 2 hours after the injection of 100 γ of biotin, the rate of citrulline synthesis is not appreciably altered, and after 3 hours it is increased only slightly above the average for the deficient group on Ration A, Table I. However, when a deficient rat was given two injections 21 hours apart, the rate of citrulline synthesis was normal 3 hours after the second injection. In a single animal, oxybiotin (O-heterobiotin) appeared not to affect the rate of citrulline synthesis 3 hours after injection.

TABLE II

Effect of Biotin Injection in Vivo on Citrulline Synthesis by Biotin-Deficient Rat Liver Homogenates

Ration fed				Time of injection prior to use	Substance injected	Citrulline per mg. N*
				hrs.		*μM*
Biotin-deficient Ration A	.		.	2	100 γ biotin	0.46
"	"	"	. .	3	100 " "	0.74
"	"	"	.	24, 3	100 " "	1.75
"	"	"	. .	2	100 " oxybiotin	0.41
Casein pair-fed control		.	. .	3	100 " biotin	2.22

* Each value represents the average of two experiments.

In preliminary experiments with control animals which had been receiving injected biotin on alternate days, it appeared that the time of the last injection of biotin influenced the level of citrulline synthesis. If the animal had not been injected for 48 hours, the level of citrulline synthesis was lower than normal and it seemed probable that the excess avidin in the gastrointestinal tract was lowering the level of biotin in the tissues. For this reason a systematic study of the effect of the time of injection of biotin upon the rate of citrulline synthesis was carried out.

The effect of varying the time of injection of biotin on the rate of citrulline synthesis in rats fed the deficient Ration A *ad libitum* and injected with 50 γ of biotin on alternate days is given in Table III. The rate apparently dropped slightly as the time after injection increased from 2 to 24 hours, although it is doubtful whether these differences are significant. The drop after 48 hours was very marked, however. It would appear from these results that ingested avidin can remove biotin from the tissues or that a continuous supply of the vitamin must be available to the tissue to maintain the normal enzyme activity.

When 80 γ of biotin were added to the incubation flasks, there was no

stimulation of the rate of citrulline synthesis by the deficient rat liver, as can be seen from Table IV. The heated extract from deficient rats did

TABLE III

Effect of Time of Biotin Injection upon Citrulline Synthesis by Biotin-Injected Control Rat Liver Homogenates

Ration fed	Time of injection* prior to use	Citrulline per mg. N†
	hrs.	*μM*
Biotin-deficient Ration A *ad libitum* + 50 γ biotin on alternate days	24, 3	1.51
	2	1.37
	3	1.21
	4	1.03
	12	0.87
	24	0.93
	48	0.55

* Each animal was given 100 γ of *d*-biotin at the time indicated. All the animals had received their last *regular* injection of 50 γ of biotin 48 hours prior to being used for experimentation.

† Each value represents the average of two experiments.

TABLE IV

Effect of Added Biotin, Heated Extract, or Heated Residue upon Citrulline Synthesis by Normal and Biotin-Deficient Rat Liver Homogenates

Liver from	Micromoles citrulline per mg. N*							
	Biotin added per flask		Heated extract added per flask			Heated residue added per flask		
	0	80 γ	0	0.5 ml., deficient	0.5 ml., control	0	0.5 ml., deficient	0.5 ml., control
Biotin-deficient rats	0.46	0.47				0.46	0.47	0.87
	0.57	0.49				0.57	0.50	0.85
			0.15	0.13	0.20			
			0.30	0.29	0.33			
			0.78	0.67	0.83			
Casein pair-fed control rats	1.40	1.20				1.40	1.12	1.21
			0.77	0.72	0.75			
			1.25	1.19	1.21			
			1.01	0.89	0.95			
Stock rat						2.24	2.25	2.24

* Each value represents the average of two experiments.

not stimulate citrulline synthesis in either the deficient or the control liver homogenates, nor did the heated residue of the deficient rat liver. However, the heated extract from control animals may have caused a very slight

stimulation of deficient liver homogenates, and *heated residue from control liver homogenates caused a considerable stimulation of citrulline synthesis by the deficient rat liver.* The rate of citrulline synthesis by the control rat liver was not stimulated by either of these control liver preparations.

The effect of a riboflavin and a pyridoxine deficiency was also investigated, with the results shown in Table V. Neither deficiency had any effect on the rate of citrulline synthesis.

TABLE V

Effect of Riboflavin and Pyridoxine Deficiency upon Citrulline Synthesis by Rat Liver Homogenates

Ration fed	Days on experiment	Weight of rat	Citrulline per mg. N*
		gm.	*μM*
Riboflavin-deficient....	62	110	1.12
"	62	103	1.56
Pyridoxine-deficient.........	70	49	1.21
"	73	105	1.39
"	73	90	1.08
Pyridoxine pair-fed control..	70	46	0.88

* Each value represents the average of two experiments

DISCUSSION

Information about the basic metabolic disturbances in a biotin deficiency is still very incomplete. It was shown in 1942 that biotin-deficient rat livers have a lowered rate of pyruvate and lactate utilization (11, 12). Later it was suggested that biotin might function as a coenzyme of CO_2 transfer (13). It has been demonstrated that aspartate can partially replace biotin for the growth of certain microorganisms (14, 15), and later studies showed that biotin was required for the fixation of CO_2 into the carbon chain from which the aspartate was formed (16–18). Other evidence points to a lowered malate oxidative decarboxylase and oxalacetate decarboxylase in biotin-deficient turkey livers (19). Paper II of this series presented data which indicate that the biotin-deficient rat is less capable of fixing CO_2 into a number of tissue components than is the normal rat (1). One of these compounds is arginine, which takes part in the Krebs-Henseleit urea cycle.

The data presented in this paper indicate that one reaction in the urea cycle which may be limiting in the biotin-deficient rat is the conversion of ornithine to citrulline. This step has been shown to involve at least two reactions in *Neurospora* (20) and evidence has been presented by Cohen and Grisolia (21) which supports the rôle of carbamyl-L-glutamate as an

intermediate in the system. Investigations are being carried on at the present time with carbamyl-L-glutamate in an attempt to locate more specifically the site of action of biotin.

Neither paired feeding procedures nor deficiencies of riboflavin or vitamin B_6 decreased the rate of citrulline synthesis. Partial inanition is therefore not responsible for the lower rate of synthesis by the biotin-deficient rat liver. The deficient rat apparently regains the normal ability to synthesize citrulline 24 hours after being given an intraperitoneal injection of 100 γ of biotin. This was a much more rapid recovery than the subsequent increase in food intake and disappearance of skin and neuromuscular symptoms. The rapidity with which injected biotin becomes effective in increasing the rate of citrulline synthesis is reminiscent of the speed with which added biotin caused a measurable uptake of $C^{14}O_2$ by cells of *Lactobacillus arabinosus* grown on a low biotin medium (18).

Free biotin added *in vitro* has proved ineffective in reversing the lowered respiration and pyruvate utilization of the cardiac muscle of biotin-deficient ducks (22) or in stimulating cell-free extracts of the aspartic deaminase of *Escherichia coli* (23), although in the latter case a stimulation was obtained when adenylic acid was added with the biotin to a freshly prepared enzyme system. The results of this investigation show that free biotin is also ineffective in reversing the lowered rate of citrulline synthesis of the biotin-deficient rat liver. The stimulation obtained with heated residue, however, provides additional evidence for the suggestion of a biotin coenzyme. It should be pointed out, nevertheless, that neither the evidence presented here nor any published at the time of this writing precludes the possibility that biotin may have a less direct function than that of a coenzyme in the several enzyme systems affected by a biotin deficiency.

SUMMARY

The synthesis of citrulline from ornithine by the washed residue of biotin-deficient rat liver homogenates is decreased about 50 per cent below that of pair-fed controls. The low rate of synthesis by the deficient liver may be increased by the addition, *in vitro*, of a heated residue of normal rat liver homogenate. It is increased to normal within 24 hours by treating the deficient animal with 200 γ of biotin given as two intraperitoneal injections. Dietary deficiencies of riboflavin or vitamin B_6 did not affect the rate of citrulline synthesis by washed liver residue.

BIBLIOGRAPHY

1. MacLeod, P. R., and Lardy, H. A., *J. Biol. Chem.*, **179**, 733 (1949).
2. Krebs, H. A., and Henseleit, K., *Z. physiol. Chem.*, **210**, 33 (1932).
3. Rittenberg, D., and Waelsch, H., *J. Biol. Chem.*, **136**, 799 (1940).
4. Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **136**, 805 (1940).

5. Grisolia, S., and Cohen, P. P., *J. Biol. Chem.*, **176,** 929 (1948).
6. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109,** 657 (1935).
7. Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **172,** 405 (1948).
8. Archibald, R. M., *J. Biol. Chem.*, **156,** 121 (1944).
9. Cohen, P. P., and Hayano, M., *J. Biol. Chem.*, **170,** 687 (1947).
10. Lardy, H. A., in Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis (1945).
11. Pilgrim, F. J., Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **145,** 237 (1942).
12. Summerson, W. H., Lee, J. M., and Partridge, W. H., *Science*, **100,** 250 (1944).
13. Burk, D., and Winzler, R. J., *Science*, **97,** 57 (1943).
14. Koser, S. A., Wright, M. H., and Dorfman, A., *Proc. Soc. Exp. Biol. and Med.*, **51,** 204 (1942).
15. Stokes, J. L., Larsen, A., and Gunness, M., *J. Biol. Chem.*, **167,** 613 (1947).
16. Lardy, H. A., Potter, R. L., and Elvehjem, C. A., *J. Biol. Chem.*, **169,** 451 (1947).
17. Shive, W., and Rogers, L. L., *J. Biol. Chem.*, **169,** 453 (1947).
18. Lardy, H. A., Potter, R. L., and Burris, R. H., *J. Biol. Chem.*, **179,** 721 (1949).
19. Ochoa, S., Mehler, H., Blanchard, M. L., Jukes, T. H., Hoffmann, C. E., and Regan, M., *J. Biol. Chem.*, **170,** 413 (1947).
20. Srb, A. M., and Horowitz, N H., *J. Biol. Chem.*, **154,** 129 (1944).
21. Cohen, P. P.. and Grisolia, S., *J. Biol. Chem.*, **174,** 389 (1948).
22. Olson, R. E., Miller, O. N., Topper, Y. J., and Stare, F. J., *J. Biol. Chem.*, **175,** 503 (1948).
23. Lichstein, H. C., *J. Biol. Chem.*, **177,** 125 (1949).

3-SUBSTITUTED THIOPHENES

IV. SYNTHESIS OF β-3-THIENYLALANINE AND ITS ANTAGONISM TO PHENYLALANINE IN THE RAT*

By ROGER G. GARST, E. CAMPAIGNE, and HARRY G. DAY

(From the Department of Chemistry, Indiana University, Bloomington)

Barger and Easson (1) synthesized β-2-thienylalanine and attempted to determine its metabolic fate in animals. Recently investigations of its "antiphenylalanine" effect have been reported by several investigators (2–5). Du Vigneaud and coworkers (2) demonstrated that the compound strongly inhibits the growth of *Saccharomyces cerevisiae*, but experiments with rats were inconclusive. Thompson and Wilkin (5) showed that β-2-thienylalanine inhibits the multiplication of the vaccinia virus in chick embryonic tissues, and that the toxicity of the thiophene derivative is neutralized by phenylalanine but not by methionine. In view of the obvious value of β-2-thienylalanine in metabolic studies, it was felt that β-3-thienylalanine should also be prepared and studied. A method of synthesis was announced (6) almost simultaneously with a report by Dittmer (7), who described a more elaborate synthesis and some of the metabolic effects of β-3-thienylalanine on *Saccharomyces cerevisiae* and *Escherichia coli*. In the report, Dittmer indicated that β-3-thienylalanine is a more potent phenylalanine antagonist than β-2-thienylalanine.

Since a study of phenylalanine antagonism in the rat seemed desirable, it was necessary to prepare a large amount of the β-3-thienylalanine. To accomplish the latter an effort was made to improve the synthesis of the antimetabolite.

Synthesis of β-3-Thienylalanine

The improved synthesis of β-2-thienylalanine reported by Dittmer, Herz, and Chambers (4) suggested a convenient synthesis of the 3 isomer. Certain modifications of the reaction were necessary, however. A flow sheet of the synthesis is given in Fig. 1. Since 3-thenyl bromide (8) is unstable and is a powerful lacrimator, it was found easiest to use the compound as the crude carbon tetrachloride solution without further distil-

lation. This gives higher yields of 3-thenylacetamidomalonic ester (III) than distilled 3-thenyl bromide, but the melting point is abnormal.[1]

Dittmer *et al.* (4) hydrolyzed 2-thenylacetamidomalonate directly to the free amino acid in 67 per cent yield with hydrobromic acid. Unfortunately this useful method cannot be employed on 3-thenylacetamidomalonate, since thiophenes which have both α positions free are extremely unstable
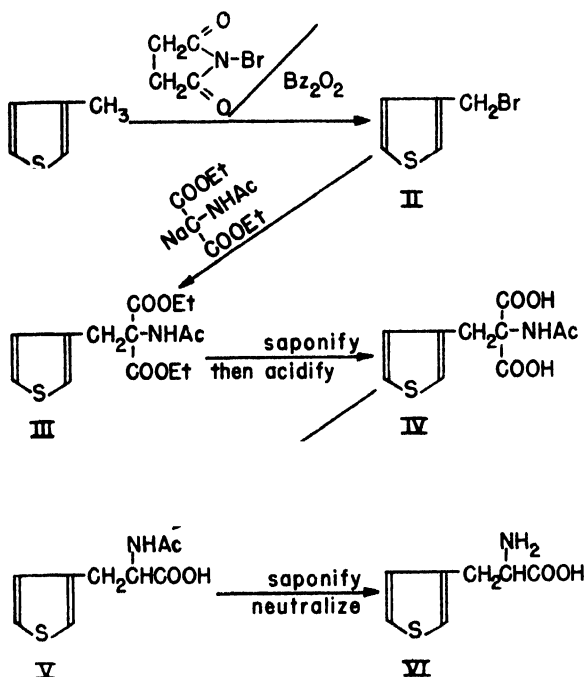
FIG. 1 Synthesis of β-3-thienylalanine

in acid solution, and polymerize to green tarry substances. Alkaline hydrolysis proceeded normally (9), saponifying the ester groups and leaving

[1] The melting point of diethyl 3-thenylacetamidomalonate was previously reported as 90–91° (6). When 3-thenyl bromide in carbon tetrachloride is allowed to react with sodioacetamidomalonic ester, the product melts from 121–130°. Melting ranges of mixtures of the two sets of crystals are intermediate. Analyses of the higher melting compound are in agreement with the formula $C_{14}H_{19}O_5NS$, and hydrolysis leads to the same compounds. Allotropism does not seem to be involved, for recrystallization from various solvents did not change the melting range. The presence of diethyl 2-bromo-3-thenylacetamidomalonate was impossible, since the compound did not contain halogen. The reason for the increase in the melting range when undistilled 3-thenyl bromide in carbon tetrachloride is used as the reagent is not clear.

the acetamido group unchanged until decarboxylation was effected. To avoid the accumulation of salts which complicate purification of the amino acid, either 3-thenylacetamidomalonic acid (IV) or preferably its less soluble decarboxylation product, N-acetyl-β-3-thienylalanine (V), was isolated from acid solution. Decarboxylation of the diacid (IV) in water required boiling for about 2 hours. Boiling for 10 minutes and then evaporating on a steam bath decarboxylated only an eighth of a sample, as shown by alkali titration.

The hydrolysis with barium hydroxide, as used previously (6), gave much lower yields than hydrolysis with sodium hydroxide, probably because of loss in removing the precipitated barium sulfate. The final hydrolysis of the N-acetyl-β-3-thienylalanine was accomplished by heating with sodium hydroxide. Following this, the solution was concentrated and the pH was adjusted to approximately 6 with hydrochloric acid. The precipitated amino acid was recrystallized from water. In this way an over-all yield of 39 per cent of β-3-thienylalanine, based on acetamido-malonic ester, was obtained.

Preparation of Diethyl 3-Thenylacetamidomalonate (III); (a) from Distilled 3-Thenyl Bromide—3-Thenyl bromide was prepared from 177 gm. (1.0 mole) of N-bromosuccinimide, as described by Campaigne and LeSuer (8) and distilled at 70–100° per 2 mm. This highly lacrimatory oil was added directly to an alcoholic solution of sodioacetamidomalonic ester prepared from 43.4 gm. (0.2 mole) of acetamidomalonic ester, and the mixture was refluxed for 3 hours. After filtering off the sodium bromide, the alcohol was removed by distillation, the oily sludge which remained was triturated with petroleum ether, and the white crystals were filtered and dried. In this way 52.1 gm. (83 per cent) of crude III were obtained. A sample recrystallized once from 50 per cent ethanol and once from 200 volumes of water melted at 90–91°.

$C_{14}H_{19}O_5NS$. Calculated, S 10.23; found, S 9.99

(b) From Crude 3-Thenyl Bromide in Carbon Tetrachloride Solution—A solution of 3-thenyl bromide in carbon tetrachloride was prepared by adding a mixture of 17.8 gm. (0.1 mole) of N-bromosuccinimide and 1.0 gm. of benzoyl peroxide batch-wise to a refluxing solution of 11 ml. (0.11 mole) of 3-methylthiophene in 50 ml. of carbon tetrachloride, cooling the mixture and filtering the succinimide. (The above modification of the bromination procedure (8) is much more rapid and seems to give higher yields.) The carbon tetrachloride solution was added directly to an alcoholic solution of 0.05 M sodioacetamidomalonic ester. A precipitate of sodium bromide appeared immediately, and after warming for 10 minutes, the solvents were removed by distillation and the residual mixture was steam-

distilled for several hours to remove oily impurities. About 50 ml. of water were allowed to accumulate in the distilling flask, which on cooling deposited 15.6 gm. of reddish solid. After being treated with activated carbon and recrystallized from ethanol, 11.7 gm. (75 per cent) of white crystals were obtained which melted from 100–115°. Recrystallization from 200 volumes of water gave white needles melting from 120–130°. This melting range was unchanged by recrystallization from dilute acid, dilute base, benzene, or 50 per cent ethylene glycol.

Calculated, S 10.23; found, S 10.18

3-Thenylacetamidomalonic Acid (IV)—In a covered beaker, 6.3 gm. (0.02 mole) of III (either Preparation *a* or Preparation *b* above gave similar yields) and 20 ml. of 10 per cent sodium hydroxide were mixed to a paste and let stand at room temperature for 24 hours, or until solution took place. The solution was filtered, cooled in ice, and neutralized with 10 ml. of 12 N hydrochloric acid, keeping the temperature below 20° to prevent decarboxylation. The precipitate thus obtained was collected, washed with water, and dried. In this way 4.3 gm. (84 per cent) of IV were obtained, which melted with the evolution of gas at 137°.

$C_{10}H_{11}O_5NS$. Calculated. S 12.46, neutral equivalent 128.6
Found. " 12.58, " " 131.8°

N-Acetyl-β-3-thienylalanine (V)—In a flask, 5.15 gm. (0.02 mole) of IV and 50 ml. of water were boiled for 2 hours, and then the solution was evaporated to near dryness over a steam bath with an aspirator to remove vapors. After cooling, the precipitate was collected, washed with a little cold water, and air-dried. There were obtained 3.6 gm. (84 per cent) of white crystals, melting at 140–144°. An analytical sample was recrystallized seven times from 10 volumes of water before achieving a melting point of 148–149°.

$C_9H_{11}O_3NS$. Calculated. S 15.03, N 6.57, neutral equivalent 213.2
Found. " 15.14, " 6.82, " " 213.7

V may be obtained directly from III without isolating IV, but in this case the alkali must be exactly neutralized with acid before boiling to prevent loss of the acid-labile thiophene derivative.

β-3-Thienylalanine (VI)—A mixture of 4.3 gm. (0.02 mole) of V and 40 ml. of 2.5 N sodium hydroxide was heated for 5 hours and allowed to evaporate to 20 ml. The cold solution was then titrated with 12 N hydrochloric acid till brom thymol blue was acidic, but Congo red was alkaline (pH between 5 and 7). After storage for 24 hours in a refrigerator, the crystals were collected and washed with ethanol and ether. This produced

2.3 gm. (67 per cent) of β-3-thienylalanine, which melted with decomposition at 265-267°.

$C_7H_9O_2NS$.   Calculated, S 18.74, N 8.19; found, S 18.43, N 8.10

Feeding Experiments

Rats—Young albino rats from a commercial breeder were used. The animals for each series of experiments were selected from shipments large enough to permit equal matching of the different groups with respect to sex, weight, and appearance. In all cases the animals were weighed and kept on the "preliminary" diet 4 days before being subdivided into the different experimental groups, thus increasing the accuracy of the matching. Individual metal cages with screen floors were used.

Diets—The basal diet was composed of casein (vitamin-free) 9, glucose (cerelose) 69, salts 4 (10); amino acid mixture 2, vitamin mixture 1, hydrogenated vegetable oil (Crisco) 15, and percomorph oil 2 drops. The amino acid mixture was composed of L-histidine hydrochloride 0.25, L-lysine hydrochloride 0.52, DL-valine 0.30, DL-threonine 0.40, DL-tryptophan 0.10, and DL-methionine 0.43. The vitamin mixture contained thiamine hydrochloride 0.055, riboflavin 0.033, nicotinic acid 0.440, pyridoxine hydrochloride 0.055, calcium pantothenate 0.550, inositol 0.550, p-aminobenzoic acid 0.550, biotin 0.001, folic acid 0.010, menadione 0.010, choline chloride 4.0, and glucose 94.0. The "preliminary" diet was the same as the basal, except that the level of casein was 18 per cent instead of 9, the amino acid mixture was omitted, and the level of the glucose was changed from 69 to 62 per cent.

The amino acid mixture was similar to the one used by Hall and Sydenstricker (11). On the basis of the data of Rose and Womack (12), it may be assumed that the phenylalanine and tyrosine level of the diet was near the minimum for normal growth. This was desirable to permit the addition of appreciable quantities of phenylalanine without inhibiting growth; for phenylalanine inhibits growth at a level only moderately higher than that necessary for optimum growth (12).

Supplements—The supplements were air-dried DL-phenylalanine, β-3-thienyl-DL-alanine, β-2-thienyl-DL-alanine, and L-tyrosine. They were thoroughly mixed with the basal diet in the amounts indicated in Table I, and all such diets were fed *ad libitum*.

Results

Over 60 animals were used. The results from most of the animals are summarized in Table I. In several instances the supplements were changed after 12 to 20 days. Several such changes are indicated in Table I.

Rats given the basal diet alone (Groups 1, 7, 12) gained an average of 28 to 35 gm. during the first 12 days. The gain was substantially the same when 0.35 per cent of DL-phenylalanine was added (Group 2), indicating that the level of phenylalanine in the basal diet was not below the

TABLE I

Antagonistic Effect of β-3-Thienylalanine and β-2-Thienylalanine on Phenylalanine

The following abbreviations are used: Pyl. for phenylalanine, β-3-Tyl. for β-3-thienylalanine, β-2-Tyl. for β-2-thienylalanine, and Tyr. for tyrosine.

Group No.	Supplement, per cent	No. of rats and sex	Weight at 4 day intervals					Gain during 12 days
			0	4	8	12	16	
			gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	None	4 M.	76	86	92	107		31
2	0.35 Pyl.	4 "	75	77	89	105		30
3	0.05 β-3-Tyl.	3 "	78	78	84	96		18
4	0.05 β-3-Tyl. + 0.35 Pyl.	4 "	76	82	90	101		25
1-a	0.30 β-3-Tyl. + 0.35 Pyl.	4 "	107	101	98	97		−10
2-a	0.35 Pyl.	4 "	105	120	130	139		34
3-a	0.15 β-3-Tyl.	3 "	96	97	103	103		7
4-a	0.15 β-3-Tyl. + 0.35 Pyl.	4 "	101	107	114	120		19
5	0.30 β-3-Tyl.	4 "	109	101	95			−14*
6	0.30 β-3-Tyl. + 0.35 Pyl.	3 "	109	99	97			−12*
7	None	4 F.	50	58	69	78	88	28
8	0.10 β-3-Tyl.	4 "	50	53	59	65	74	15
9	0.10 β-2-Tyl.	4 "	50	55	65	74	81	24
10	0.10 β-2-Tyl. + 0.35 Pyl.	4 "	50	55	66	81	92	31
11	0.10 β-3-Tyl. + 0.35 Pyl.	3 "	54	60	70	81	93	27
9-a	0.10 β-3-Tyl.	4 "	81	84	89	93		12
10-a	0.10 β-3-Tyl. + 0.35 Pyl.	4 "	92	105	113	121		29
12	None	4 "	75	86	96	110	119	35
13	0.175 Tyr.	4 "	75	86	96	107		32
14	0.10 β-3-Tyl.	4 "	75	70	73	81	87	6
15	0.10 β-3-Tyl. + 0.175 Tyr.	4 "	76	76	79	87	92	11
16	0.10 β-3-Tyl. + 0.35 Tyr.	2 "	67	69	72	78		5*
17	0.10 β-3-Tyl. + 0.35 Tyr.	2 "	93	97	101			8*
18	0.35 Tyr.	2 "	65	78	90			25*

* Gain during 8 days.

minimum for satisfactory growth, and that at least 0.35 per cent could be added without decreasing the growth rate.

β-3-Thienylalanine decidedly impaired growth (Groups 3, 3-a, 5, 8, 14), even at the level of 0.05 per cent. DL-Phenylalanine caused a decisive reversal of the effect at low levels of β-3-thienylalanine, but when the level of the antagonist was 0.30 per cent, loss of weight occurred, even though the diet contained 0.35 per cent of added phenylalanine.

Although β-2-thienylalanine inhibited growth, when fed at the level of 0.1 per cent, the effect was decidedly less than in the case of β-3-thienyl-alanine fed at the same level (Groups 7 to 10). The inhibition was reversed with DL-phenylalanine.

Owing to the ability of tyrosine to reduce the phenylalanine requirement (13), it was of interest to determine the effect of tyrosine on the growth of rats fed β-3-thienylalanine. In the experiments reported, L-tyrosine was used at the levels of 0.175 and 0.35 per cent. At the lower level the tyrosine definitely did not effect any degree of reversal. Over a period of 16 days on the supplements the average gain of four rats given 0.10 per cent β-3-thienylalanine was 12 gm. Four well matched controls given 0.175 per cent tyrosine, in addition to the β-3-thienylalanine, were limited to the same gain in weight. Also, when tyrosine was fed at the level of 0.35 per cent and the β-3-thienylalanine at 0.10 per cent (Groups 16 and 17, Table I) there was no indication of reversal in the growth inhibition of the β-3-thienylalanine.

The effect of tyrosine on the toxicity of β-2-thienylalanine was not investigated.

In addition to growth retardation, the rats supplemented with β-3-thienylalanine alone had rough and unkempt fur and an abject appearance. This was not so apparent in the rats receiving β-2-thienylalanine as the only supplement.

<div align="center">DISCUSSION</div>

The apparent failure of L-tyrosine to reverse the growth inhibition of β-3-thienylalanine is in agreement with the finding of Dittmer et al. (3), who reported that L-tyrosine had no effect on the inhibitory action of β-2-thienylalanine on the growth of yeast, although DL-phenylalanine was effective. This indicates that the β-3-thienylalanine competes with phenylalanine for some essential enzyme system in the rat other than that which converts phenylalanine to tyrosine. This is contrary to the interpretation of Beerstecher and Shive (14), who regard their results with β-2-thienylalanine and *Escherichia coli* as evidence that the latter analogue competes with phenylalanine for the system which oxidizes phenylalanine to tyrosine.

It is unfortunate that the data afford no evidence concerning the effect of the D isomer of phenylalanine in reversing the toxicity of the thienyl-alanines. Rats can use this isomer approximately as efficiently as the L isomer for growth (12). Thus it is probable that the D isomer is capable of preventing the growth inhibition of the thienylalanines, but the possibility should be investigated. In an article (15) which was published after the submission of this paper it was shown that both β-2-thienyl-D-alanine

and β-2-thienyl-L-alanine inhibit the growth of rats, and that the effect is reversed with DL-phenylalanine.

SUMMARY

1. The synthesis of β-3-thienylalanine is described.

2. β-3-Thienylalanine inhibits the growth of rats on a diet containing little if any excess of phenylalanine. The effect is reversed with DL-phenylalanine, but not with L-tyrosine.

3. β-3-Thienylalanine is a more potent antagonist of phenylalanine than β-2-thienylalanine in the rat.

BIBLIOGRAPHY

1. Barger, G., and Easson, A. P. T., *J. Chem. Soc.*, 2100 (1938).
2. du Vigneaud, V., McKennis, H., Jr., Simmonds, S., Dittmer, K., and Brown, G. B., *J. Biol. Chem.*, **159**, 385 (1945).
3. Dittmer, K., Ellis, G., McKennis, II., Jr., and du Vigneaud, V., *J. Biol. Chem.*, **164**, 761 (1946).
4. Dittmer, K., Herz, W., and Chambers, J. S., *J. Biol. Chem.*, **166**, 541 (1946).
5. Thompson, R. L., and Wilkin, M. L., *Proc. Soc. Exp. Biol. and Med.*, **68**, 434 (1948).
6. Campaigne, E., Bourgeois, R. C., Garst, R., McCarthy, W. C., Patrick, R. L., and Day, H. G., *J. Am. Chem. Soc.*, **70**, 2611 (1948).
7. Dittmer, K., Abstracts, Division of Biological Chemistry, American Chemical Society, Chicago, 113th meeting, April (1948); *J. Am. Chem. Soc.*, **71**, 1205 (1949).
8. Campaigne, E., and LeSuer, W. M., *J. Am. Chem. Soc.*, **70**, 1555 (1948).
9. Snyder, H. R., and Smith, C. W., *J. Am. Chem. Soc.*, **66**, 350 (1944).
10. Jones, J. H., and Foster, C., *J. Nutr.*, **24**, 245 (1942).
11. Hall, W. K., and Sydenstricker, V. P., *Arch. Biochem.*, **12**, 147 (1947).
12. Rose, W. C., and Womack, M., *J. Biol. Chem.*, **166**, 103 (1946).
13. Womack, M., and Rose, W. C., *J. Biol. Chem.*, **166**, 429 (1946).
14. Beerstecher, E., Jr., and Shive, W., *J. Biol. Chem.*, **167**, 49 (1947).
15. Ferger, M. F., and du Vigneaud, V., *J. Biol. Chem.*, **179**, 61 (1949).

STUDIES ON THE MECHANISM OF NITROGEN STORAGE

I. EFFECTS OF ANTERIOR PITUITARY GROWTH HORMONE PREPARATIONS ON PLASMA GLUTAMINE, TOTAL FREE AMINO ACIDS, AND THE EXCRETION OF URINARY AMMONIA

By PAUL D. BARTLETT, OLIVER H. GAEBLER, AND ANNE HARMON

(From the Edsel B. Ford Institute for Medical Research, Henry Ford Hospital, Detroit)

Although glutamine constitutes from 15 to 25 per cent of the total free amino acids of plasma and is a major constituent of many animal tissues (1), little is known concerning its utilization in the animal body. A function clearly established, however, and one which certainly might be sufficient to explain its presence as a major constituent, is its rôle in the transport and storage of ammonia and hence in the regulation of acid-base economy (2). Suggestion has also been made that glutamine might function as an interim storage and transport form of amino nitrogen (3). This would seem to be a reasonable speculation, since the amide nitrogen of glutamine is particularly labile, and, in addition, its removal results in the release of glutamic acid with a similarly active amino group.

Early in the development of metabolic studies, in which crude extracts of growth hormone were used and which were characterized by the production of both nitrogen storage and gain of body weight in dogs, observations were made on the blood chemistry. Teel and Watkins (4) observed a drop of 20 to 30 per cent in the blood non-protein nitrogen and found that not more than 70 per cent of the decrease could be accounted for by changes in urea and amino acids. Gaebler (5) confirmed the finding of a drop in non-protein nitrogen and found in addition a decrease in the excretion of total urinary nitrogen largely accounted for by changes in the excretion of urea. The urinary output of ammonia also increased during the 48 hours immediately following injection. Farr and Alpert (6), using both the intravenous and intraperitoneal routes of administration of crude growth hormone extracts, observed respectively an increase and a decrease in plasma amino acid level.

Since we had at our disposal growth preparations which were very active when assayed in rats, but which were known to produce miscellaneous effects on nitrogen storage in dogs (7), it seemed of interest to study in particular their effect on the level of plasma glutamine and the excretion of urinary ammonia, and to reexamine the changes produced in plasma amino acids in the dog.

Animals—Adult female dogs weighing from 15 to 18 kilos were used in all experiments. The animals were maintained in metabolism cages and on Stock Diet 6, previously described (7).

Methods—Urine collections were made in 24 hour periods terminated by catheterization and washing of the bladder. Total urine nitrogen was determined by a semimicro modification of the Kjeldahl-Gunning pro-

TABLE I

Effects of Anterior Pituitary Growth Hormone Preparations on Body Weight, Total Urinary Nitrogen, Ammonia Excretion, and Plasma Glutamine (Dog 42)

Day of experiment	Body weight	Urine		Plasma glutamine
		Total N	Ammonia N	
	kg.	*gm. per 24 hrs.*	*gm. per 24 hrs.*	*mg. per cent*
1-5*	15.51	9.61	0.56	13.7
6†	15.62	11.05	0.52	9.2
7	15.86	7.81	0.50	10.0
8	16.08	5.74	0.59	15.9
9	16.08	6.98	0.64	17.3
10	15.86	8.15	0.53	17.5
11	16.08	8.50	0.45	17.9
12	16.19	8.97	0.50	13.0
13	15.92	9.34	0.52	14.5
14	15.92	11.18	0.62	15.3
15	15.86	11.62	0.68	13.7
16	15.86	10.84	0.61	14.3
17	15.86	10.89	0.45	12.4
18	15.86	11.05	0.52	14.5
19	15.86	10.24	0.53	14.0

* Data recorded for 5 day control period are the averages of daily analyses.

† Data recorded for this day are for the 24 hour period following subcutaneous injection of 200 mg. of growth hormone preparation, Parke, Davis and Company, Rx099816, which assayed 1361 units per gm. in normal, adult female rats.

cedure with 0.02 N acid and alkali. Urinary ammonia was determined by the Van Slyke and Cullen aeration procedure (8). Plasma glutamine levels were determined by the enzymatic method of Archibald (9), and plasma total free amino acids by the ninhydrin method of Hamilton and Van Slyke (10). Fasting specimens drawn upon completion of a 24 hour urine collection were used in all blood analyses.

Growth Hormone Preparations—Assayed growth hormone preparations were supplied by reliable commercial pharmaceutical manufacturers.[1]

[1] We are especially indebted to Dr. D. A. McGinty and Mr. L. W. Donaldson of Parke, Davis and Company, and to Dr. John R. Mote of the Armour Laboratories for supplying assayed preparations.

The lyophilized powders were dissolved in 0.8 per cent saline and injected subcutaneously.

Results

Table 1 summarizes the results of one of our early experiments showing the effects of growth hormone on body weight, total urinary nitrogen, ammonia excretion, and plasma glutamine. During the first 24 hour period following injection, an increase in the excretion of urinary nitrogen and a sharp drop in plasma glutamine were observed. An anabolic phase

TABLE II

Effects of Anterior Pituitary Growth Hormone Preparations on Body Weight, Total Urinary Nitrogen, Ammonia Excretion, and Plasma Glutamine (Dog 45)

Day of experiment	Body weight	Urine		Plasma glutamine
		Total N	Ammonia N	
	kg.	*gm. per 24 hrs.*	*gm. per 24 hrs.*	*mg. per cent*
1–5*	17.55	10.42	1.09	12.5
6†	18.21	11.55	0.73	7.9
7	17.87	9.32	1.28	14.2
8	17.76	9.26	0.99	15.0
9	17.76	9.95	No analysis	13.7
10	17.76	10.75	0.97	12.3
11	17.76	11.50	0.73	
12	17.76	11.54	0.68	11.0
13	17.65	11.52	0.72	
14	17.65	11.96	0.71	10.0
15	17.65	11.71	0.70	
16	17.65	11.82	0.66	
17	17.53	11.82	0.68	10.4
18–20	17.53 (Average)	11.43	0.76 (Average)	
21	17.31	11.37	0.59	10.3

* Data recorded for 5 day control period are the averages of daily analyses.

† Data recorded for this day are for the 24 hour period following subcutaneous injection of 200 mg. of growth hormone preparation, Armour Laboratories, lot 3-PKR-3. This preparation produced a 20 gm. weight increase in hypophysectomized rats injected with 50 γ per day for a 15 day period.

characterized by several periods of gain in weight, nitrogen storage, and an elevation of plasma glutamine followed. In general, the excretion of urinary ammonia followed changes in plasma glutamine and was largest during periods of plasma glutamine elevation.

Table II summarizes data obtained in a similar experiment conducted on another animal with a different growth hormone preparation. Although the preparation showed high growth hormone activity in the rat, an entirely different response was produced in the dog. The period of

protein anabolism was of relatively short duration and of small magnitude, whereas the protein catabolic phase was prolonged and severe in respect to nitrogen loss. Opportunity was thus presented for a study of the inverse relationship of plasma glutamine to total urinary nitrogen excretion under conditions of greatly stimulated protein catabolism. Changes in the total urinary nitrogen and ammonia excretion and the level of plasma glutamine followed the same general pattern observed in the experiment summarized in Table I. Plasma glutamine values during the catabolic

TABLE III

Effects of Anterior Pituitary Growth Hormone Preparations on Body Weight, Total Urinary Nitrogen, Ammonia Excretion, Plasma Glutamine Amide Nitrogen, and Total Free Amino Acid Carboxyl N (Dog 45)

Day of experiment	Body weight	Urine		Plasma	
		Total N	Ammonia N	Glutamine amide N	Free amino acid carboxyl N
	kg.	*gm. per 24 hrs.*	*gm per 24 hrs.*	*mg. per cent*	*mg per cent*
1-5*	19.16	10.52	0.59	1.17	5.09
6†	19.52	10.43	0.42	0.79	5.00
7	19.41	8.74	0.72	1.20	7.26
8	19.30	8.80	0.70	1.29	6.45
9	19.41	9.75	0.67	1.27	5.58
10	19.30	10.30	0.73	1.56	0.89‡
11	19.30	11.20	0.70	1.20	4.61
12	19.18	11.40	0.54		
13	19.18	12.27	0.65	1.11	4.74
14	18.95	12.53	0.68	1.18	4.65

* Data recorded for 5 day control period are the averages of daily analyses.

† Data recorded for this day are for the 24 hour period following subcutaneous injection of 200 mg. of growth hormone preparation, Parke, Davis and Company. Rx099858, which assayed 1780 units per gm. in normal, adult female rats.

‡ Though recognized as most unusual, the value for free amino acid carboxyl nitrogen on the 10th day of the experiment is not due to error in analysis or calculation.

phase were uniformly lower than those obtained for the control period and were accompanied by an increased excretion of total urinary nitrogen.

Results of a third experiment in which both the plasma glutamine amide nitrogen and the free amino acid carboxyl nitrogen were followed are shown in Table III. A significant elevation in the amino acid carboxyl nitrogen was obtained and observed to reach a peak value during the second 24 hour period immediately following injection. It should be noted that the peak elevation in plasma glutamine was also obtained, but that it occurred at the onset of a marked catabolic phase indicated by amino acid breakdown.

Changes in glutamine amide nitrogen of plasma did not on any experimental day account for changes observed in the total free amino acid carboxyl nitrogen.

DISCUSSION

Our finding of an elevation in plasma amino acids in the dog following subcutaneous injection of anterior pituitary growth hormone preparations of the type described is confirmatory of a similar observation reported by Farr and Alpert (6) following intravenous administration of crude extracts, but contrary to those reported by Teel and Watkins (4) and Farr and Alpert (6) in which crude extracts were administered intraperitoneally.

In recent studies on normal rats reported by Li *et al.* (11), a reduction in the level of plasma amino acids was obtained following injection of 1 mg. of purified growth hormone. Their observations were made up to 12 hours after administration of the preparation and, although a reduction in the plasma amino acid level was observed at 6 hours, the level had returned to normal at the end of 12 hours. In hypophysectomized rats, considered to be more sensitive to growth hormone than the normal rat (12), the plasma amino acid level was found to be significantly elevated above the control level 6 hours after treatment.

It seems quite apparent from the results of our studies that changes in plasma glutamine amide nitrogen do not account for changes observed in the total free amino acid carboxyl nitrogen or for the nitrogen stored. If the increase in plasma-free amino acid carboxyl nitrogen had been effected through the removal of amide or amino nitrogen from glutamine, some evidence for the rôle of glutamine in the interim storage of amino nitrogen would have been obtained. The fact that glutamine amide nitrogen reaches its highest value during the period of marked amino acid catabolism is in complete agreement with the rôle of glutamine in the transport and storage of ammonia.

A point of considerable interest and one which is difficult to explain is the sharp drop in plasma glutamine which occurred during the first 24 hour period immediately following injection of growth hormone. If the enzymatic synthesis of glutathione is catalyzed by anterior pituitary growth hormone (13), and glutamine is involved, then perhaps the sudden drop in the plasma glutamine level could be explained on this basis. The direct incorporation of glutamine through the amide grouping into tissue proteins might also explain our finding.

SUMMARY

1. Changes in plasma glutamine do not account for changes observed in plasma amino acids or for nitrogen stored following administration of growth hormone.

2. Close parallelism in the level of plasma glutamine and the excretion of urinary ammonia was observed in both anabolic and catabolic phases of the experiments and emphasizes again the important rôle of glutamine in the transport and storage of ammonia.

3. An anterior pituitary growth hormone preparation shown to produce nitrogen storage and gain of weight in the dog also produced an elevation in the plasma amino acid level.

BIBLIOGRAPHY

1. Hamilton, P. B., *J. Biol. Chem.*, **158**, 397 (1945).
2. Van Slyke, D. D., Phillips, R. A., Hamilton, P. B., Archibald, R. M., Futcher, P. H., and Hiller, A., *J. Biol. Chem.*, **150**, 481 (1943).
3. Braunstein, A. E., *Advances in Protein Chem.*, **3**, 42 (1947).
4. Teel, H. M., and Watkins, O., *Am. J. Physiol.*, **89**, 662 (1929).
5. Gaebler, O. H., *J. Exp. Med.*, **57**, 349 (1933).
6. Farr, L. E., and Alpert, L. K., *Am. J. Physiol.*, **128**, 772 (1939–40).
7. Bartlett, P. D., and Gaebler, O. H., *Endocrinology*, **43**, 329 (1948).
8. Van Slyke, D. D., and Cullen, G. E., *J. Am. Med. Assn.*, **62**, 1558 (1914).
9. Archibald, R. M., *J. Biol. Chem.*, **154**, 643 (1944).
10. Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, **150**, 231 (1943).
11. Li, C. H., Geschwind, I., and Evans, H. M., *J. Biol. Chem.*, **177**, 91 (1949).
12. Li, C. H., and Evans, H. M., in Harris, R. S., and Thimann, K. V., Vitamins and hormones, New York, **5**, 200 (1947).
13. Goss, H., and Gregory, P. W., *Proc. Soc. Exp. Biol. and Med.*, **32**, 681 (1935).

EFFECT OF CYSTINE AND THREONINE ON THE GROWTH OF RATS RECEIVING TRYPTOPHAN-DEFICIENT RATIONS*

BY L. V. HANKES, L. M. HENDERSON,† AND C. A. ELVEHJEM

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

A suppression of the growth rate of rats upon addition of proteins or protein hydrolysates containing little or no tryptophan to 9 per cent casein diets supplemented with cystine has been repeatedly demonstrated (1–5). This effect can be duplicated by adding 0.078 per cent threonine or 0.208 per cent phenylalanine to such diets (6). With 1.5 mg. per cent of niacin or 50 mg. per cent of tryptophan growth rates are restored to values higher than those obtained without added threonine or phenylalanine.

Cystine supplementation aggravates the renal damage and fatty infiltration of the liver of rats receiving 8 per cent casein diets, deficient in choline (7, 8). Griffith and Nawrocki (9) have reported further intensification of the choline deficiency when threonine was added with cystine. The influence of threonine was believed to be due not to a direct antagonistic action on choline or on labile methyl, but rather to a stimulation of growth or metabolism, which aggravated the existing choline deficiency. They suggested that sulfur-containing amino acids and threonine are the most limiting amino acids for the rat receiving an 8 per cent casein diet. In most of the studies of tryptophan-niacin deficiency low casein diets, supplemented with cystine, have been employed. The aggravation of choline deficiency by threonine, in the presence of supplementary cystine, and a somewhat similar aggravation of a niacin deficiency by threonine, also in the presence of added cystine, suggested that the cystine was of some importance in the latter case. The experiments reported here establish the relationship of cystine or methionine to threonine in precipitating a tryptophan-niacin deficiency in the rat.

The experimental procedures were similar to those employed in previous studies (1–3, 6). Sprague-Dawley rats, 3 weeks of age, kept in individual cages, were fed *ad libitum* for a 5 week period and weighings were made at weekly intervals. The basal diet had the following composition: sucrose 81.8 per cent, casein[1] 9 per cent, Salts IV (10) 4 per cent, corn oil 5 per cent, L-cystine 0.2 per cent, and vitamins added as a dry mixture diluted with sucrose to provide the following quantities per 100 gm. of diet: thiamine 0.2 mg., riboflavin 0.3 mg., pyridoxine 0.25 mg., calcium *dl*-pantothenate 2 mg., choline chloride 100 mg., inositol 10 mg., biotin 0.01 mg., and folic acid 0.02 mg. Halibut liver oil diluted with corn oil and fortified with vitamins E and K was used as a source of the fat-soluble vitamins. This was fed by dropper once weekly to provide approximately the following daily intakes: vitamin A 400 I.U., vitamin D 4 I.U., 2-methyl-1,4-naphthoquinone 0.06 mg., and α-tocopherol 0.7 mg. A ration containing 0.2 per cent L-cystine was used as the control in order that the results of the experiments repoited in this paper may be compared to those in earlier papers of this series. The average growth rate for fifteen negative control animals was 12 gm. per week, and for fifteen positive control animals (basal plus 1.5 mg. of niacin) was 16 gm. per week in a series of five experiments reported As in previous work, the growth inhibition appeared after the animals were on the experiment for at least 2 weeks (6).

In two experiments urine was collected under toluene from animals of selected groups after the 5th week. The collections were made for 3 day periods before and after the administration of 300 mg. of DL-tryptophan by stomach tube. The urine samples were analyzed for N^1-methylnicotinamide by the method of Huff *et al.* (11). A portion of each sample was neutralized, diluted, and analyzed for nicotinic acid by microbiological assay (12). The values obtained were designated "free" niacin. Another portion of each sample was hydrolyzed by autoclaving for 1 hour at 15 pounds pressure with an equal volume of 2 N HCl. After neutralizing and diluting, "total"[2] niacin was determined microbiologically (12). The identity of the substance which gives rise to niacin activity for *Lactobacillus arabinosus* during acid treatment has been identified as quinolinic acid by Henderson (13).

In several experiments the 0.2 per cent L-cystine was removed from the ration to determine its rôle in the production of a growth inhibition. In

---

[1] Smaco purified casein.

[2] Unpublished data of L. M. Henderson shows that the acid treatment used decarboxylates only a small, but presumably constant portion of the quinolinic acid present.

some cases 0.2 per cent DL-methionine was substituted for the 0.2 per cent L-cystine.

<div align="center">TABLE I</div>

Effect of Certain Supplements on Growth of Rats Receiving Low Niacin Diets

Supplement	L-Cystine	DL-Methionine	Without niacin		Plus 1.5 mg. niacin per 100 gm. ration	
			No. of animals	Average gain, per cent of negative controls	No. of animals	Average gain, per cent of positive controls
	per cent	*per cent*				
None		0.2	3	110	3	97
"			3	92	3	64
0.078% L-threonine	0.2		6	18		
0.078% DL-threonine	0.2		6	42	3	111
0.156% "	0.2		6	9		
0.078% "		0.2	6	35	6	107
0.078% "			6	98	6	57
0.156% "			3	91		
0.312% "			3	66		
0.052% D-phenylalanine	0.2		3	63		
0.104% "	0.2		3	58		
0.208% L-phenylalanine	0.2		6	126	3	92
0.208% DL-phenylalanine	0.2		12	57	3	106
0.208% "		0.2	6	68	6	110
0.208% "			6	82	6	60
0.208% DL-phenylalanine + 0.078% DL-threonine	0.2		6	42		
0.208% DL-phenylalanine + 0 078% DL-threonine		0.2	6	29		
0.208% DL-phenylalanine + 0.078% DL-threonine			6	106		
2% glycine	0.2		9	56	3	107
2% "		0.2	6	63	6	96
2% "			9	76	6	70
2% acid-hydrolyzed casein	0.2		9	47	6	135
2% " "		0.2	6	47	6	112
2% " "		0.4	3	26	3	128
2% " "			9	164	9	91

RESULTS AND DISCUSSION

The effect of selected amino acids or acid-hydrolyzed casein on the growth of rats receiving the basal ration with and without niacin is shown in Table I. The growth rates are presented as percentages of the growth

of the negative control group or of the positive control group in the same experiment. The criteria used for evaluating growth-inhibition are the same as those employed in earlier studies in this laboratory (3, 6).

Since previous work had shown that threonine and phenylalanine were the amino acids present in acid-hydrolyzed casein (6) responsible for the growth inhibition, the activity of the isomers of these acids was determined. As shown in Table I, 0.078 per cent L-threonine[3] and 0.104 per cent D-phenylalanine were found to be very active growth inhibitors, while 0.104 per cent L-phenylalanine had no effect under these conditions.
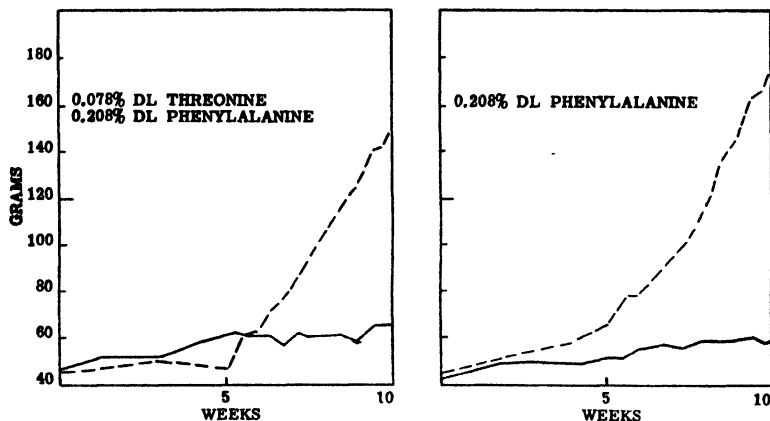
Fig. 1. Growth response of rats to supplementary tryptophan. The dotted line is the growth curve of an animal receiving 20 mg. of DL-tryptophan daily by mouth, after the first 5 weeks. The continuous line is the growth curve of an animal not supplemented with tryptophan.

Removal of the added 0.2 per cent L-cystine from the ration relieved the growth inhibition in all cases. Replacement of the 0.2 per cent L-cystine with 0.2 per cent DL-methionine caused a strong growth inhibition in the presence of 0.078 per cent DL-threonine and 2 per cent acid-hydrolyzed casein.[4] DL-Phenylalanine or glycine was a little less effective when methionine was added as the sulfur-containing amino acid. In all cases, threonine or acid-hydrolyzed casein caused a much stronger growth inhibition than either glycine or phenylalanine. The fact that 0.2 per cent L-cystine or 0.2 per cent DL-methionine caused a growth inhibition in the presence of 0.208 per cent DL-phenylalanine, 0.078 per cent DL-threonine, or 2 per cent acid-hydrolyzed casein indicated a definite relationship between phenylalanine, cystine or methionine, threonine, and tryptophan.

[3] We are indebted to Dr. W. C. Rose of the University of Illinois for the L-threonine.

[4] Smaco casein was hydrolyzed with sulfuric acid as described previously (6).

Daily supplementation of selected groups of animals with 20 mg. of DL-tryptophan for an additional 5 weeks relieved the growth inhibition caused by 0.078 per cent DL-threonine, 2 per cent glycine, or 0.208 per cent DL-phenylalanine in the presence of either 0.2 per cent L-cystine or 0.2 per cent DL-methionine. This indicated that tryptophan was a very

TABLE II

Effect of Various Substances on Excretion of Nicotinic Acid and Its Derivatives by Rats Following Tryptophan Administration

Experiment No.	Supplement	Urinary excretion, γ per 24 hrs. per 100 gm. body weight											
		Free nicotinic acid				Total nicotinic acid following acid hydrolysis				N¹-Methylnicotinamide			
		Control*		Tryptophan		Control*		Tryptophan		Control*		Tryptophan	
		Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range
I	None	6	5–6	60	40–77	11	9–12	409	97–935	8	6–10	641	376–992
	Niacin	12	9–15	71	50–98	18	17–23	1377	801–1817	53	32–63	639	506–767
	2% acid-hydrolyzed casein	6	5–6	56	49–68	7	7–8	1403	1233–1570	7	6–7	463	369–518
	Same + niacin	10	9–11	73	68–80	14	13–15	963	619–1482	16	11–18	689	467–918
II	None	7	5–11	36	22–47	11	9–13	945	700–1080				
	Niacin	6	3–11	240	49–545	11	9–15	919	543–1149				
	0.078% DL-threonine	4	3–5	30	20–46	7	7–8	779	154–1372				
0	Same + niacin	7	5–9	132	55–187	13	13–16	1047	734–1421				
	No cystine + 0.078% DL-threonine + 0.2% DL-methionine	7	6–8	30	30–30	10	8–11	1052	862–1243				
	Same + niacin	7	5–8	54	42–61	11	9–14	1387	1044–1471				

*The control refers to the 3 day collection prior to feeding DL-tryptophan.

limiting factor in these rations. A few typical growth curves obtained in these experiments are presented in Fig. 1. In all cases those animals receiving a supplement of tryptophan for the second 5 week period grew at a more rapid rate than the control animals.

The analytical data (Table II) from urine of selected groups of animals in two different experiments indicated that the addition of 2 per cent acid-

hydrolyzed casein had no depressing effect on the excretion of N^1-methyl-nicotinamide. However, the addition of niacin to the ration significantly increased the N^1-methylnicotinamide levels in the urine of both groups. The level of "free" niacin in the urine of control animals was not affected significantly by the addition of 2 per cent acid-hydrolyzed casein, 0.078 per cent DL-threonine, or 0.078 per cent DL-threonine plus 0.2 per cent DL-methionine. There were no significant differences in the "total"[2] niacin excreted by the animals in the different groups after tryptophan administration. This indicated that in the presence of excessive amounts of tryptophan any possible interference with niacin synthesis was probably overcome by the mass effect of one component (tryptophan) in the system.

It is possible that the inhibition obtained with threonine in the presence of cystine could be due to some reaction occurring in the intestinal tract or tissues between threonine, cystine, and tryptophan, which utilizes some of the tryptophan, leaving insufficient amounts for niacin and protein synthesis. The mode of action of these inhibiting amino acids is not known at the present. although injection experiments have shown that threonine inhibits growth whether injected or fed in the ration, while phenylalanine inhibits only when fed (6).

Studies with enterectomized rats have indicated that most of the synthesis of niacin and related compounds from large doses of tryptophan occurs in the tissues (14, 15), but it was recognized that physiologically significant quantities of the vitamin might be synthesized in the intestinal tract as suggested previously (16). Additional evidence of tissue synthesis has been reported by several workers (17, 18).

With the thought in mind that threonine, cystine, or phenylalanine may cause growth inhibition by interference with niacin synthesis, tissue slice work was done to test the effect of these compounds. It was found by microbiological assay of acid-hydrolyzed liver slices that the niacin content increases on an average of 30 per cent after a 40 minute incubation period.[5] The range of increase obtained was 20 to 70 per cent. These increases were obtained by incubation of slices without the addition of any amino acids. The addition of threonine, phenylalanine, cystine, or combinations of these amino acids to the incubation solutions had no effect on the levels of niacin in the incubated slices. Other organs were tested for ability to synthesize niacin and it was found that similar increases in niacin content occurred in kidney slices after a 40 minute incubation period. No increases in niacin content were found after incubation of intestinal tract and muscle slices. It should be emphasized that the increases in niacin were obtained without the addition of tryptophan to the incubated solutions, and that the niacin values obtained probably represent the sum of

[5] Hankes, L. V., unpublished data.

free niacin and niacin due to decarboxylation of quinolinic acid (if present), during acid hydrolysis.

Since the inhibiting amino acids had no effect on the niacin levels of incubated liver slices or the level of niacin excreted in the urine of animals receiving these added amino acids, it appeared that a metabolic interrelationship between these amino acids was the important factor in this work. Several amino acids are limiting in a 9 per cent casein ration, when calculated on the basis of Rose's suggested minimal levels for normal rat growth (19). Those most limiting are methionine, histidine, threonine, lysine, phenylalanine, and tryptophan. The growth rates obtained with
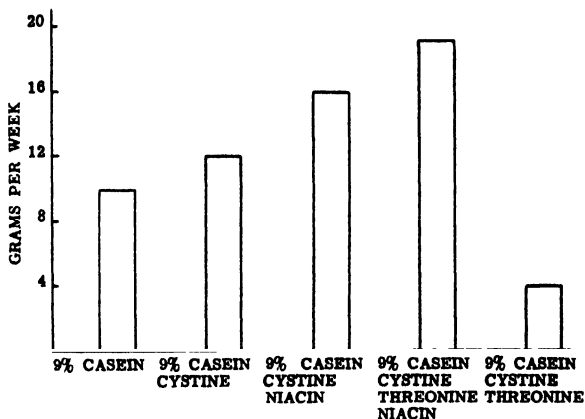
FIG. 2. The cystine, threonine, and niacin interrelationships on 9 per cent casein rations.

the different combinations of amino acids are illustrated in Fig. 2. The growth on a 9 per cent casein ration without L-cystine was 10 gm. per week. The limiting amino acids in this case were cystine, threonine, and tryptophan. The addition of cystine to the ration increased the growth rate up to 12 gm. per week, and left threonine and tryptophan as limiting amino acids. The addition of niacin, which has a sparing effect on tryptophan, increased the growth rate up to 16 gm. per week, and the only limiting amino acid remaining on this ration was threonine. The further addition of threonine increased the growth to 19 gm. At this point the omission of niacin from the ration again made tryptophan more limiting, and growth dropped to 4 gm. per week. Whether similar results can be obtained with another protein limiting in tryptophan and other amino acids has not been determined. Thus a tryptophan deficiency accompanied by adequate or generous intake of threonine and cystine is much more serious than when these amino acids are supplied at low levels.

Such interrelationships have often been observed in vitamin deficiencies. It is well known in vitamin work that clear cut symptoms due to the lack of a single vitamin are not obtained until all of the other factors are supplied in sufficient quantities. For example, Dye et al. (20) in their work with multiple vitamin deficiencies showed that low levels of vitamin A and thiamine in the ration were not as damaging as when one of them was added in excess.

Experiments (Table III) in which the level of protein was varied indicated that the growth inhibition obtained was not due merely to the presence of free amino acids. Replacement of 2 per cent of the protein in the ration with amino acids equivalent to 2 per cent casein produced almost

TABLE III

Effect of Protein Level on Growth Rate

Level of protein	Glycine	Without niacin	Plus 1.5 mg. niacin per 100 gm. ration
		Average gain, per cent of negative controls (6 rats)	Average gain, per cent of positive controls (6 rats)
	per cent		
7% casein + amino acids ≅ 2% casein		80	96
9% " + " " ≅ 2% " (except trypto-phan)		30	125
7.5% casein		73	67
7.5% "	2	29	50
10.5% "		115	135
10.5% "	2	57	121

the same growth as a 9 per cent casein basal diet. Furthermore, the addition of 2 per cent casein to this ration (tryptophan excluded) gave a very strong growth inhibition. Since this procedure was equivalent to adding 2 per cent of acid-hydrolyzed casein to the basal ration, it indicated that the inhibition was due to an imbalance of amino acids in the ration. Reduction of the protein level of the ration from 9 to 7.5 per cent increased the inhibitory activity obtained with 2 per cent glycine. However, the elevation of the level of protein to 10.5 per cent did not affect the growth rates to any significant extent in the presence of 2 per cent glycine.

It is of interest that all of the inhibitors used except 6 per cent gelatin did not inhibit growth when dextrin was used as a source of carbohydrate (6). The cause of this effect is not known, but it is possible that the slower digestion of dextrin allows intestinal bacteria more time for synthesis of niacin.

SUMMARY

1. L-Threonine and D-phenylalanine are the isomers responsible for the growth-inhibitory effect of these amino acids when added to a 9 per cent casein ration containing no niacin.

2. The omission of 0.2 per cent L-cystine from the ration relieved the growth inhibition due to 0.078 per cent DL-threonine, 2 per cent acid-hydrolyzed casein, 0.208 per cent DL-phenylalanine, and 2 per cent glycine.

3. The addition of 0.2 per cent DL-methionine in place of 0.2 per cent L-cystine in a 9 per cent casein ration gave the same growth effect as 0.2 per cent L-cystine in the presence of either 0.078 per cent DL-threonine or 2 per cent acid-hydrolyzed casein.

4. The addition of the growth-inhibiting amino acids to a 9 per cent casein ration did not affect the excretion of niacin in the urine of rats.

5. The growth inhibitions obtained by adding the various combinations of amino acids appear to be due to the increased requirement of the limiting amino acid when all others are supplied in adequate or generous amounts.

BIBLIOGRAPHY

1. Krehl, W. A., Sarma, P. S., and Elvehjem, C. A., *J. Biol. Chem.*, **162,** 403 (1946).
2. Krehl, W. A., Henderson, L. M., de la Huerga, J., and Elvehjem, C. A., *J. Biol. Chem.*, **166,** 531 (1946).
3. Henderson, L. M., Deodhar, T., Krehl, W. A., and Elvehjem, C. A., *J. Biol. Chem.*, **170,** 261 (1947).
4. Singal, S. A., Sydenstricker, V. P., and Littlejohn, J. M., *J. Biol. Chem.*, **176,** 1063 (1948).
5. Rosen, F., and Perlzweig, W. A., *J. Biol. Chem.*, **177,** 163 (1949).
6. Hankes, L. V., Henderson, L. M., Brickson, W. L., and Elvehjem, C. A., *J. Biol. Chem.*, **174,** 873 (1948).
7. Mulford, D. J., and Griffith, W. H., *J. Nutr.*, **23,** 91 (1942).
8. Salmon, W. D., *J. Nutr.*, **33,** 155 (1947).
9. Griffith, W. H., and Nawrocki, M. F., *Federation Proc.*, **7,** 288 (1948).
10. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109,** 657 (1935).
11. Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **167,** 157 (1947).
12. Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. and Eng. Chem., Anal. Ed.*, **15,** 471 (1943).
13. Henderson, L. M., *J. Biol. Chem.*, **178,** 1005 (1949).
14. Henderson, L. M., and Hankes, L. V., *Proc. Soc. Exp. Biol. and Med.*, **70,** 26 (1949).
15. Hundley, J. M., *Federation Proc.*, **8,** 386 (1949).
16. Teply, L. J., Krehl, W. A., and Elvehjem, C. A., *Am. J. Physiol.*, **148,** 91 (1947).
17. Schweigert, B. S., German, H. L., and Garber, M. J., *J. Biol. Chem.*, **174,** 383 (1948).
18. Hurt, W. W., Scheer, B. T., and Deuel, H. J., Jr., *Arch. Biochem.*, **21,** 87 (1949).
19. Griffith, J. Q., Jr., and Farris, E. J., The rat in laboratory investigation, Philadelphia, 83 (1942).
20. Dye, M., Porter, T., and Kelly, E., *J. Nutr.*, **33,** 459 (1947).

METABOLISM OF EMULSIFIED TRILAURIN (—C¹⁴OO—) AND OCTANOIC ACID (—C¹⁴OO—) BY RAT TISSUE SLICES*

By R. P. GEYER, L. W. MATTHEWS, and F. J. STARE

(*From the Department of Nutrition, Harvard School of Public Health, and the Department of Biological Chemistry, Harvard Medical School, Boston*)

Relatively few studies concerned with the metabolism of the longer fatty acids (C_{12} and upwards) *in vitro* have been done with tissue slices because of the low water solubility of non-toxic forms of these compounds and the lack of suitable analytical methods to detect accurately the small amount of metabolism which occurs during such experiments. At present, however, it is possible to overcome these difficulties by the use of non-toxic, finely dispersed emulsions of triglycerides containing radioactive fatty acids labeled with C^{14}. By this means, sufficient substrate can gain access to the cells, and it or its metabolic products can be followed by measurement of the radioactivity. That highly emulsified triglycerides probably enter tissue cells without difficulty is indicated by the extremely rapid rate at which emulsified radioactive trilaurin was metabolized when injected intravenously into rats (1).

The studies reported in this paper deal with the ability of slices of various rat tissues to metabolize aerobically carboxyl-labeled trilaurin in an emulsified form and octanoic acid (—C¹⁴OO—) as its sodium salt. The latter was used in part to ascertain whether certain phenomena found with the trilaurin could be attributed to the physical state of the substrate. Utilization of the lipides was measured by the amount of radioactivity in the respired carbon dioxide.

EXPERIMENTAL

Preparation of Radioactive Trilaurin and Octanoic Acid—Lauric acid containing C^{14} in the carboxyl group was prepared in 75 to 86 per cent yields from undecyl magnesium bromide and $C^{14}O_2$ according to the method of Harwood and Ralston (2). The product had a correct neutral equivalent and a melting point of 43.6–44.0° (uncorrected). It was diluted with 4 parts of purified inert lauric acid (melting point of the mixture, 43.5–44.0°, uncorrected) and then synthesized to trilaurin by the method of Garner

(3). A petroleum ether solution of the product was thoroughly extracted with dilute aqueous potassium bicarbonate and then repeatedly with 20 per cent ethyl alcohol. The yields ranged from 71 to 80 per cent based on the activity of the lauric acid. The trilaurin used in the studies reported in this paper had the correct saponification number and an activity of 920 counts per minute per mg.[1] The activity of the trilaurin was obtained by counting it directly when plated out in stainless steel cups, with or without lens paper disks.

Carboxyl-labeled octanoic acid was prepared from heptyl magnesium bromide and $C^{14}O_2$ by the technique used in the lauric acid syntheses. The acidified reaction mixture was extracted with ether, and the ether solution was washed well with H_2O, and then with dilute NaOH solution. The alkaline solution was extracted with ether, acidified with H_2SO_4, and extracted with ether. The solvent was evaporated to give octanoic acid with a correct neutralization equivalent and in a yield of 72 per cent based on activity. When counted directly as its sodium salt with the aid of lens paper disks, it had an activity of 6580 c.p.m. per mg. Synthetic inactive sodium octanoate was used as a diluent when necessary.

Emulsification of Trilaurin—1.5 gm. of the labeled trilaurin, 0.2 gm. of a soy bean phospholipide preparation (Preparation B(F-2), Geyer *et al.* (4)), 0.1 gm. of Demal-14,[2] and 28 ml. of water were blended at high speed in a 50 ml. metal blendor jar equipped with a thermometer and an inlet for gassing with nitrogen. The blending was carried out for 20 minutes and during this time the temperature was raised to 90° by external heating. At the end of this period most of the particles were below 1 μ in diameter and none were above 2 μ as judged by visual microscopy with a calibrated ocular micrometer. The emulsion was quickly taken up in a syringe and transferred to 1 ml. glass ampules which were flooded with nitrogen and then sealed in a flame. These were then autoclaved at 15 pounds for 15 minutes and stored in the dark. Although such emulsions are stable for long periods of time, no emulsions were used when more than 1 month old.

Studies with Rat Tissues in Vitro—These were carried out as follows: 125 ml. conical flasks were used for the incubations and were equipped in such a way as to allow CO_2-free oxygen saturated with water to bubble through the medium and then either (*a*) through a gas-washing tube and through a sintered glass disk into a measured quantity of 1 N CO_2-free NaOH, or (*b*) through a by-pass into a large alkaline trap. The organs from

---

[1] All counting was done with an end window Geiger-Mueller tube, and all counts were corrected to what would have been obtained had 5 mg. been present during counting.

[2] A polyglycerol ester of oleic acid. Emulsol Corporation, Chicago.

a lightly etherized rat were excised and placed in oxygenated calcium-free Ringer-phosphate solution (5) at 0°. Slices were then made and placed in the incubation flasks which contained 20 ml. of the Ringer's solution at 37.5° and pH 7.1. The flasks were gassed at a rapid rate for 3 to 5 minutes, then while the substrate was being added, and then for an addi-
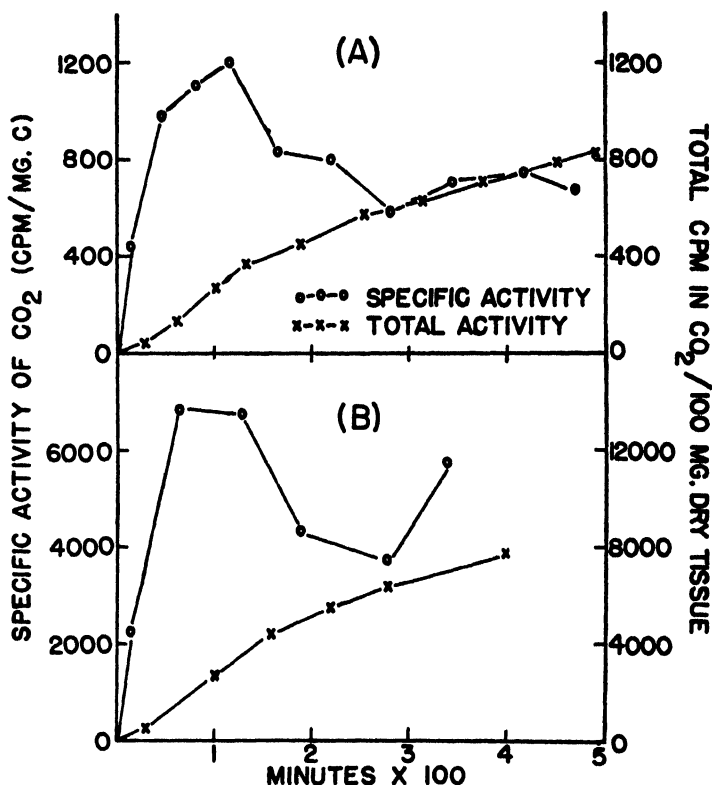
FIG. 1. Metabolism of carboxyl-labeled lipides by liver slices. *A*, trilaurin (—$C^{14}OO$—) metabolism. Initial activity, 33,500 c.p.m.; 920 c.p.m. per 1 mg. of trilaurin. *B*, octanoic acid (—$C^{14}OO$—) metabolism. Initial activity, 93,600 c.p.m.; 6500 c.p.m. per 1 mg. of octanoic acid.

tional 1 to 2 minutes. During this preliminary period the oxygen and respired carbon dioxide were diverted through the by-pass (*b*). The by-pass was then closed and the gases from the flasks were passed through their respective columns of alkali at a rate of approximately 5 ml. per minute. Slow mechanical shaking was provided. The alkaline samples of respired carbon dioxide were collected directly in 25 ml. volumetric flasks without exposure to atmospheric carbon dioxide, and aliquots were

analyzed for total CO_2 in the Van Slyke-Neill apparatus (6). Suitable aliquots were also treated with $BaCl_2$ solution and the precipitated $BaCO_3$ washed and plated as described by Solomon *et al.* (7).

Since the oxidation *in vivo* of intravenously administered trilaurin was extremely rapid (1), it was desirable in the studies *in vitro* to ascertain first whether or not whole rat blood or plasma played a rôle in this metabolism, or whether the tissues were able to assimilate the dispersed lipide
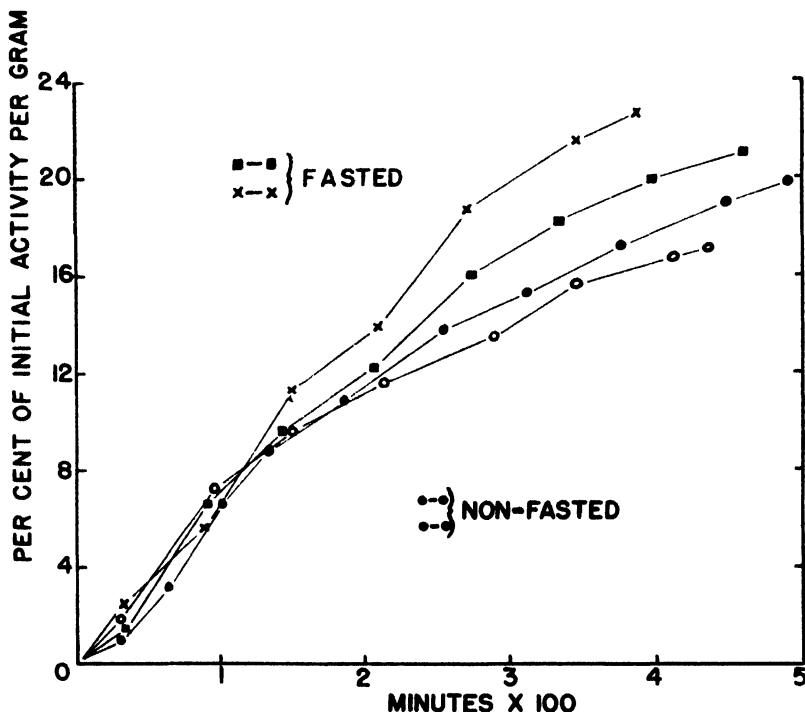
FIG. 2. Metabolism of trilaurin by liver slices. Per cent of initial trilaurin activity which appeared in the respired CO_2 per gm. of dry liver. The initial activity in each experiment was 33,500; 920 c.p.m. per 1 mg. of trilaurin.

quickly without previous physical or chemical change. Preliminary studies disclosed that heparinized whole rat blood or plasma from such blood had a negligible effect upon the trilaurin, while intact liver slices oxidized this substrate equally well when the suspending medium was either of these materials or the Ringer-phosphate solution. Thus, blood components such as lipases seemed unnecessary for the metabolism of the triglyceride and any differences in the amount of blood retained by the slices of various organs could be neglected.

Prolonged Incubation Studies with Liver—These studies were carried out to determine both the ability of such slices to metabolize the labeled lipides and the duration of such metabolism in surviving slices. In early experiments 1 to 2 gm. (dry weight) of liver slices were used, but this was later reduced to 0.2 to 0.5 gm. Either 36 mg. of radioactive trilaurin in the form of the emulsion or 14.4 mg. of octanoic acid (as the sodium salt) were used per flask. Successive samples of respired carbon dioxide were collected at various intervals for total experimental periods ranging up to

TABLE I

*Effect of Fasting Upon Octanoate (—C¹⁴OO—) Oxidation in Vitro by Various Tissues**

Tissue	Fast period	Dry tissue	Specific activity of CO_2†	Total c.p.m. in CO_2 per 100 mg. dry tissue
	hrs.	*c.mm. CO_2 per mg.*		
Liver	0	9.25	1609	796
	36	5.36	1907	552
	60	6.99	1806	676
Kidney	0	25.35	4770	6510
	36	23.40	4735	5940
	60	22.30	5545	6640
Heart	0	9.21	2020	998
	36	8.90	1857	686
	60	10.20	1012	552
Lung	0	11.18	5210	3130
	36	8.90	3920	1870
	60	9.62	4350	2250
Brain	0	11.00	1031	609
	36	9.51	1320	674
	60	12.60	1142	769

* 2 hour incubation period. All flasks contained 0.001 M octanoic acid as its sodium salt. Total activity per flask, 18,720 c.p.m.

† Counts per minute per mg. of carbon in carbon dioxide.

7 hours. The results of typical experiments are given in Fig. 1 in the form of time curves for the specific activity of the respired carbon dioxide and the accumulated activity which appeared in the CO_2.

Effect of Fasting—To determine whether or not fasting influenced the rate and extent of trilaurin metabolism by liver, experiments were undertaken in which the animals were fasted for various periods of time prior to sacrificing them. Approximately 1 gm. of liver slices (dry weight) was used in each experiment, and the incubations were performed as described previously. The results from some of these studies are presented in Fig. 2 where the per cent of the original activity present in the respired

carbon dioxide is plotted against time. Little effect of fasting was evident during the first 2 hours of incubation, and further experimentation is necessary to evaluate the significance of the slight differences which occurred after this time.

TABLE II

Octanoate Oxidation by Various Tissues at Various Substrate Concentrations*

Tissue	Octanoic acid concentration	Dry tissue	Specific activity of CO_2	Total c.p.m. in CO_2 per 100 mg dry tissue	Total c.p.m. in CO_2 per 100 mg. dry tissue per mM substrate
	mM per l.	*c.mm. CO_2 per mg.*			
Brain	0.3	11.8	790	500	1670
	1.0	11.0	1,030	609	675
	3.3	8.64	1,400	649	196
	6.6	8.00	1,750	680	108
Liver	0.3	10.3	611	337	1120
	1.0	9.25	1,610	796	885
	3.3	6.76	4,460	2,880	872
	6.6	5.75	8,320	5,740	910
Lung	0.3	11.4	3,120	1,900	6330
	1.0	11.2	5,210	3,130	3480
	3.3	9.94	5,430	2,895	878
	6.6	8.28	7,360	3,420	542
Heart	0.3	13.9	934	697	2320
	1.0	9.21	2,020	998	1110
	3.3	12.9	3,200	2,320	703
	6.6	11.8	8,370	5,150	543
Kidney	0.3	25.1	1,910	2,530	8430
	1 0	25.4	4,770	6,510	7230
	3.3	19.6	10,660	9,320	2820
	6.6	22.1	13,580	13,550	2150
Spleen	0.3	9.19	3,390	1,670	5570
	3.3	10.6	6,700	3,800	1150
	6.6	9.49	7,320	3,720	590

* 2 hour incubation. Each flask contained 20 ml. of Ca-free Ringer-phosphate solution and 0.3 ml. of H_2O which contained the proper amount of octanoic acid (as its sodium salt) to give a final substrate concentration of 0.3, 1.0, 3.3, or 6.6 mM per liter. Total activity per mM of octanoic acid per flask, 18,720 c.p.m.

The effect of fasting was also studied in a series of experiments in which sodium octanoate was the substrate. Slices of various rat tissues (liver, lung, kidney, spleen, heart, and brain) from animals fasted for varying lengths of time were incubated with 0.001 M radioactive sodium octanoate for 2 hour periods, and the total and specific activities of the respired carbon dioxide were determined. The results are presented in Table I and

show no appreciable increase in octanoate metabolism as a result of fasting; instead, some decreases in total octanoate oxidation were observed. In view of these results and those obtained with the trilaurin, non-fasted animals were used in the succeeding experiments reported in this paper. The data in Table I show a marked difference between organs with respect to their ability to oxidize octanoic acid.

Effect of Concentration of Substrate—Studies were undertaken to determine whether the concentration of the substrate influenced the amount of oxidation by the various organs. Octanoic acid concentrations of 0.0003, 0.0033, and 0.0066 M were used, and approximately 0.2 to 0.3 gm.

TABLE III

*In Vitro Metabolism of Emulsified Trilaurin (—$C^{14}OO$—) by Various Tissues**

Tissue	Dry tissue	Specific activity of CO_2	Total c.p.m. in CO_2 per 100 mg. dry tissue
	c.mm. CO_2 per mg.		
Liver	7.24	199	77.4
	7.04	238	89.8
Kidney	15.7	799	681
	16.2	696	605
Heart	10.7	499	287
	11.81	384	244
Lung	9.41	427	216
	10.00	527	282
Spleen	8.52	518	236
	8.71	437	190
Skeletal muscle	3.61	69.7	13.5
	3.34	49.8	8.9
Brain	6.95	84.9	31.4

* 2 hour incubation period. Each flask contained 16 mg. of trilaurin in the form of an emulsion. Total activity per flask, 14,800 c.p.m.

of tissue (dry weight) was used in each case. The incubations were of 2 hours duration and the respired carbon dioxide was collected as in the previous experiments and assayed for radioactivity. The results of these studies are given in Table II. The data show that the metabolism of octanoic acid by slices of rat tissue depends to a certain extent upon the level of substrate present, and that, as a consequence, the relative ability of these organs to metabolize this acid is also dependent upon the concentration of substrate present.

The ability of slices of various rat tissues to oxidize trilaurin was studied with the organs previously mentioned and also with skeletal muscle. 16 mg. of trilaurin in the form of the emulsion were added to each flask and

the incubations were conducted for 2 hour periods. The results are given in Table III and are similar to those obtained with the higher levels of octanoic acid.

DISCUSSION

By using a finely dispersed emulsion of radioactive trilaurin it was possible to study the metabolism of this triglyceride by rat tissue slices *in vitro*. Since comparable results were obtained with water-soluble sodium octanoate, the use of such emulsions appears to be a valid and useful tool for the study of fat metabolism by such slices. The importance of the use of such emulsions lies in the fact that most of the water-soluble forms of the fatty acids which contain 12 or more carbon atoms are relatively toxic to surviving cells, while the non-toxic forms are so water-insoluble as to have difficulty in entering the cell. The high sensitivity and accuracy with which the radioactivity can be determined allow even small amounts of oxidation to be detected and measured.

In the studies reported in this paper the radioactivity which appeared in the respired carbon dioxide was used as the criterion of metabolism of the marked substrate. This simple index is a measure only of completely oxidized substrate (specifically only the completely oxidized carboxyl groups of the lipides) and does not, of course, give evidence as to how the metabolism is accomplished or to what extent intermediates have accumulated or entered into intermediary metabolism. Furthermore, it is possible that a higher rate of metabolism was observed than would be the case if the labeled carbon had been farther back in the chain, or if fatty acids other than lauric and octanoic were employed. These various aspects are now under study.

It can be seen from Figs. 1 and 2 that trilaurin and octanoate metabolism is most rapid in the first 2 hours, but that the metabolism is maintained at a significant rate for as long as 4 to 5 additional hours. The increasing specific activity of the respired carbon dioxide during the first 120 minutes may be explained in part by the possibility that some substrates naturally present in the liver are being exhausted (or perhaps less efficiently oxidized), with the net result that less inert CO_2 is being formed to act as a diluent. Additional studies on the two-phase character of the oxidation-time curve will be reported in a subsequent paper. The data in Table I and Fig. 2 indicate that for the studies reported in this paper there would have been no advantage in using fasted animals, since no appreciable increase in metabolism of the lipides resulted from such a procedure. The slightly greater rate of oxidation of trilaurin that occurred after the first 2 hours of incubation in the case of the liver slices from fasted animals compared to that from non-fasted animals needs further study.

The relative order of the various tissues with respect to their ability to oxidize octanoic acid is dependent to a certain extent upon the level of substrate used. Thus, at the lowest level of this acid (0.0033 M) brain oxidized more of this acid than did liver, whereas at higher levels of substrate liver oxidized much more than brain. On the basis of the amount oxidized per mM of substrate present, liver was not affected by increasing the substrate concentration in spite of a decrease in total CO_2 production. The efficiency of all of the other organs to oxidize octanoic acid rapidly fell off as the substrate concentration was raised. This is of interest from an *in vivo* standpoint, since in normal animals after oral ingestion of fat the liver is subjected to a much higher concentration of lipide than are the other organs. If the various tissues have the same relative ability to oxidize these lipides *in vivo* as they do *in vitro*, it would appear that extrahepatic tissues as well as the liver are not only important in the utilization of the split fragments from fatty acid catabolism, but are also of significance in the metabolism of the longer chain fatty acids themselves.

These studies have been extended to include the effects of various catalysts and inhibitors and will be reported in a subsequent paper.

SUMMARY

Carboxyl-labeled trilaurin in a finely emulsified form was metabolized by kidney, liver, spleen, heart, lung, brain, and skeletal muscle slices as measured by the radioactivity in the respired carbon dioxide. Water-soluble sodium octanoate gave results similar to those obtained with the trilaurin emulsion.

Metabolism of these substrates proceeded most rapidly during the first 2 hours, but significant oxidation occurred for as long as 6 to 7 hours in liver tissue.

The relative order of the ability of the various organs to oxidize octanoic acid depended to a certain extent upon the level of substrate present.

BIBLIOGRAPHY

1. Geyer, R. P., Chipman, J., and Stare, F. J., *J. Biol. Chem.*, **176**, 1469 (1948).
2. Harwood, H. J., and Ralston, A. W., *J. Org. Chem.*, **12**, 740 (1947).
3. Garner, T. L., *J. Soc. Chem. Ind.*, **47**, 278 T (1928).
4. Geyer, R. P., Mann, G. V., Young, J., Kinney, T. D., and Stare, F. J., *J. Lab. and Clin. Med.*, **33**, 163 (1948).
5. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 194 (1947).
6. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
7. Solomon, A. K., Gould, R. G., and Anfinsen, C. B., *Phys. Rev.*, **72**, 1097 (1947).

THE ADRENAL CORTEX AND SERUM PEPTIDASE ACTIVITY*

By THEODORE B. SCHWARTZ† and FRANK L. ENGEL

(*From the Department of Medicine, Duke University School of Medicine, Durham, North Carolina*)

Studies have been initiated in this laboratory to investigate the relation of various hormones to proteolytic enzyme activity. This report deals with the effect, in the rat, of adrenal cortical extract (ACE) and adrenocorticotropic hormone (ACTH) on the enzymic hydrolysis of L-leucylglycylglycine (LGG) by rat serum. A smaller series of mice has been similarly studied, since Holman, White, and Fruton (1) have reported a considerable increase in mouse serum peptidase activity after injection of ACE and ACTH.

Materials and Methods

Adult male rats of the Vanderbilt strain, fed a diet of dog chow supplemented with liver pudding and lettuce (2), were used. Water intake was not restricted, but, prior to bleeding, the rats were fasted 18 or 40 hours. Animals were subjected to light nembutal anesthesia during the course of bleeding from the tail, vena cava, or aorta. No change in enzymic activity of the blood could be related to the site of bleeding. Most of the serum samples were not hemolyzed and all samples showing more than a trace of hemolysis were discarded.

Adult male mice, fed an unrestricted diet of dog chow without supplements, were bled from the vena cava after ether anesthesia was induced.

Aqueous adrenal cortical extract[1] and a purified preparation of adrenocorticotropic hormone[2] were administered subcutaneously or intraperitoneally.

Enzymic hydrolysis was carried out in 10 ml. Erlenmeyer flasks continuously agitated in a Warburg bath kept at 37°. The hydrolysis mixture, maintained at pH 8 with 0.02 M veronal buffer, contained, in addition, 0.05 mM of LGG,[3] 0.2 ml. of serum, and 0.85 per cent NaCl to make a total

volume of 2 ml. When desired, the mixture was made 0.001 M with respect to manganese sulfate without changing its total volume.

At measured time intervals after the initiation of the hydrolysis, 0.2 or 0.5 ml. samples were withdrawn from the hydrolysis mixture. The hydrolytic products were assumed to be L-leucine and glycylglycine (3, 4). The assumption that only 1 peptide bond is broken seems reasonable, since, calculated on this basis, we have found 102 per cent hydrolysis of LGG in 24 hours. The free amino acid content was determined by the manometric ninhydrin method of Van Slyke and associates (5–7). Substrate solutions usually were prepared at weekly intervals and occasional checks failed to reveal the presence of free amino acid. Hydrolysis mixtures prepared with serum but without substrate were used as controls for each determination of enzyme activity and all procedures, including the enzymic hydrolyses, were performed in duplicate.

Results

As indicated by representative examples in Fig. 1, the hydrolysis of LGG by rat serum apparently conforms to the kinetics of a zero order reaction until more than 50 per cent of the substrate is hydrolyzed. Therefore, until approximately half of the substrate is hydrolyzed, the enzymic (LGGase) activity of a known quantity of serum may be reflected quantitatively by a constant, K^0_{LGG}, the per cent hydrolysis of LGG per minute.

No attempt was made to relate K^0_{LGG} to serum protein concentration. However, that such a linear relationship may exist is indicated by the fact that halving or doubling the serum concentration in the hydrolysis mixture resulted in a corresponding halving or doubling of the value of K^0_{LGG}. Therefore, by using 0.1 ml. of serum per ml. of hydrolysis mixture throughout this study, K^0_{LGG} may be taken as a measure of enzyme concentration and is defined here as the *per cent hydrolysis per minute for 0.1 ml. of serum per ml. of hydrolysis mixture.* In the studies that follow, K^0_{LGG} routinely was determined at 30 and 60 minutes for each serum sample.

Mean control K^0_{LGG} values under various conditions are shown in Table I. In rats fasted 18 hours prior to bleeding, K^0_{LGG} averaged 0.34 ± 0.022 in 30 minutes and 0.31 ± 0.019 in 60 minutes. In the presence of 0.001 M manganese sulfate, there was a striking increase in the LGG-splitting ability of rat serum, K^0_{LGG} reaching a mean value of 0.88 ± 0.032 in 30 minutes and 0.86 ± 0.037 in 60 minutes. This increase is highly significant statistically (P values all less than 0.01). The K^0_{LGG} values for animals fasted for 40 hours were not significantly different from those of animals fasted 18 hours.

The effect of ACE on LGGase activity of the serum of 18 hour fasted rats was investigated by determining K^0_{LGG} on serum samples obtained
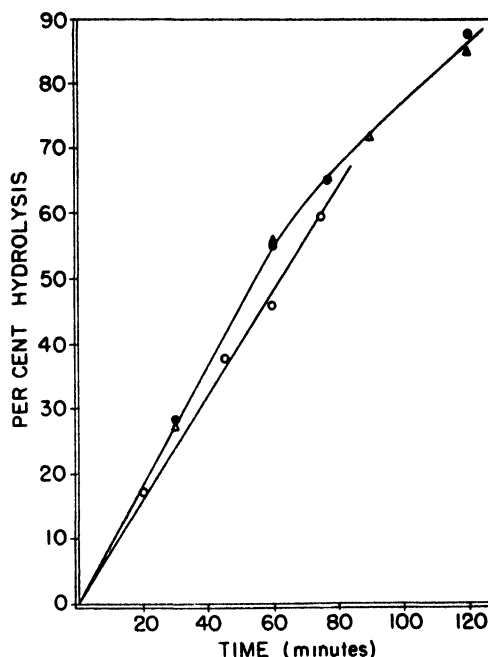
FIG. 1. Hydrolysis of L-leucylglycylglycine by normal rat serum. These hydrolyses were performed in the presence of 0.001 M MnSO₄ as activator. Each symbol (O, ●, and △) represents the serum of a single rat. Hydrolysis was performed as indicated in the text.

TABLE I

Hydrolysis of L-Leucylglycylglycine by Fasted Rat Serum

Period of fasting	Hydrolysis	Mean K^0_{LGG}*		$P\dagger$
		No activator	0.001 M MnSO₄	
hrs.	*min.*			
18	30	0.34 ± 0.022‡ (6)§	0.88 ± 0.032 (9)	<0.01
	60	0.31 ± 0.019 (5)	0.86 ± 0.037 (7)	<0.01
40	30	0.43 ± 0.031 (4)	0.78 ± 0.053 (4)	<0.01
	60	0.38 ± 0.023 (4)	0.70 ± 0.036 (4)	<0.01

* Determined for 0.1 ml. of serum per ml. of hydrolysis mixture.
† Determined from Fisher's table (9).
‡ Standard error of the mean.
§ The figures in parentheses indicate the number of observations.

both immediately preceding and 4 to 8 hours following the intraperitoneal injection of ACE (2 ml. per 100 gm. of body weight). Thus, each animal served as its own control. The data in Table II indicate that there was

no significant increase in mean K^0_{LGG} values 4 to 8 hours after the administration of ACE. It is of interest that this group includes three animals, each of which received 8 ml. of ACE over an 8 hour period, and still no

TABLE II

Effect of Adrenal Cortical Extract on LGGase Activity of Rat* Serum

Activator	Post-injection interval	Hydrolysis	No. of observations	Mean K^0_{LGG}*		P‡	Remarks
				Before injection	After injection†		
	hrs.	*min.*					
None	4	30	3	0.39 ± 0.004	0.39 ± 0.071	>0.5	
		60	3	0.33 ± 0.029	0.43 ± 0.068	0.2	
	8	30	4	0.35 ± 0.018	0.45 ± 0.078	0.2	2 animals of this group given 8 ml. ACE
		60	4	0.33 ± 0.020	0.40 ± 0.050	0.1	
0.001 M MnSO₄	4	30	5	0.90 ± 0.038	1.26 ± 0.299	0.3	1 animal ill (see text)
		60	4	0.85 ± 0.059	1.00 ± 0.181	>0.5	
	8	30	1	0.88 ± 0.032§	0.89		This animal given 8 ml. ACE
		60	1	0.86 ± 0.037§	0.93		

* All the animals were fasted for 18 hours prior to initial determination of K^0_{LGG}. Each rat served as his own control.

† Unless otherwise stated, each rat received ACE, 2 ml. per 100 gm., body weight, intraperitoneally.

‡ Calculated from the mean difference of K^0_{LGG} for each group of rats.

§ Control values of rats fasted 18 hours (Table I).

TABLE III

Effect of Adrenocorticotropic Hormone on LGGase Activity of Rat Serum

Activator	Hydrolysis	Control group; 4 observations	Injected group*		P
		Mean K^0_{LGG}	No. of observations	Mean K^0_{LGG}	
	min.				
None	30	0.43 ± 0.031	9	0.50 ± 0.031	0.1
	60	0.38 ± 0.023	7	0.45 ± 0.030	0.1
0.001 M MnSO₄	30	0.78 ± 0.053	9	0.91 ± 0.052	0.1
	60	0.70 ± 0.036	8	0.85 ± 0.055	0.1

* Rat weights ranged from 100 to 200 gm. and each animal was bled 8 hours after the injection of 2 mg. of ACTH.

appreciable increase over control values was observed. One animal, rather obviously ill, showed a 117 per cent increase in LGGase activity 4 hours after the administration of ACE, 2 ml. per 100 gm. of body weight. Despite this isolated instance, the mean increase in K^0_{LGG} in this group remained insignificant.

The influence of ACTH, similarly employed, reflects that of ACE. After a 40 hour fast, 100 to 200 gm. rats were given 2 mg. of ACTH subcutaneously 8 hours before serum samples were taken. Values for K^0_{LGG} were compared with those of untreated rats fasted 40 hours. Again, as indicated in Table III, the difference between control values and those observed 8 hours after ACTH injection is statistically invalid.

Finally, the effect of ACE on mice was investigated. Fed mice were bled 4 hours after intraperitoneal injection of 1 ml. of ACE, and K^0_{LGG} of serum, pooled from two or three animals, was determined. Table IV shows these K^0_{LGG} values compared with a series of untreated controls. Once more, there is essentially no difference in mean K^0_{LGG} values of the two groups. These sera exhibited varying degrees of hemolysis and it was noted that the three serum pools which showed no hemolysis had the

TABLE IV

Effect of Adrenal Cortical Extract on LGGase Activity of Pooled* Mouse Serum

Activator	Hydrolysis	Control group; 6 observations	Injected group;† 7 observations	P
		Mean K^0_{LGG}	Mean K^0_{LGG}	
	min.			
None	30	0.33 ± 0.049	0.38 ± 0.030	0.3
	60	0.32 ± 0.034	0.33 ± 0.018	>0.5

* Each determination was made on sera pooled from two or three mice.

† Each mouse received 1 ml. of ACE 4 hours prior to bleeding.

lowest K^0_{LGG} values, averaging 0.23. Furthermore, it was our impression that the degree of hemolysis was roughly proportional to increments in K^0_{LGG} over the values for non-hemolyzed serum pools.

DISCUSSION

The enzymic hydrolysis of LGG by rat serum is quite similar to that observed with guinea pig serum (4) and mouse serum (1), in that, under the conditions employed, zero order reaction kinetics apparently are operative and the enzymic activity is greatly enhanced in the presence of manganese sulfate.

We have been unable to demonstrate that the parenteral administration of ACE or ACTH to rats results in any appreciable increase in serum LGGase activity. On the contrary, if we exclude the activating effect of manganese sulfate, it is our impression that this peptidase activity of non-hemolyzed rat serum tends to remain at a relatively constant level. In addition, we have found that mouse serum LGGase activity is completely uninfluenced by treatment of mice with ACE. These findings differ considerably from those of Holman, White, and Fruton (1), who

reported increases up to 272 per cent in LGGase activity in the serum of mice given doses of ACE and ACTH comparable to those used in this study. We can offer no adequate explanation for our failure to confirm their results.

The correlation between the degree of hemolysis present in mouse serum samples and the increment in value of K^0_{LGG} is of interest and immediately suggests that LGGase activity is present in mouse erythrocytes. Indeed, we[4] have found that K^0_{LGG} for washed, packed mouse erythrocytes has a mean value of 5.3 for 0.1 ml. of packed erythrocytes per ml. of hydrolysis mixture. Thus, per unit volume, the concentration of LGGase activity in mouse erythrocytes is 20 times that in mouse serum. Recently, Johansen and Thygersen (8) have shown that human erythrocytes contain 50 times as much LGGase activity as human serum. Findings such as these emphasize the necessity to avoid hemolysis in making determinations of serum K^0_{LGG}.

SUMMARY

The initial rate of enzymic hydrolysis of L-leucylglycylglycine by rat serum has been found to conform to the kinetics of a zero order reaction. In the presence of manganese sulfate this peptidase activity was doubled.

The administration of adrenal cortical extract or adrenocorticotropic hormone to rats was not followed by any appreciable change in peptidase activity of unhemolyzed rat serum. Similarly, the serum peptidase activity of mice given adrenal cortical extract was found to be essentially the same as that of untreated control mice.

Increases in mouse serum peptidase activity appeared to be correlated with the degree of hemolysis of serum samples.

BIBLIOGRAPHY

1. Holman, H. R., White, A., and Fruton, J. S., *Proc. Soc. Exp. Biol. and Med.*, **65**, 196 (1947).
2. Engel, F. L., Schiller, S., and Pentz, E. I., *Endocrinology*, **44**, 458 (1949).
3. Smith, E. L., and Bergmann, M., *J. Biol. Chem.*, **153**, 627 (1944).
4. Fruton, J. S., *J. Biol. Chem.*, **166**, 721 (1946).
5. Van Slyke, D. D., Dillon, R. T, MacFadyen, D. A., and Hamilton, P. B., *J. Biol. Chem.*, **141**, 627 (1941).
6. MacFadyen, D. A., *J. Biol. Chem.*, **145**, 387 (1942).
7. Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, **150**, 231 (1943).
8. Johansen, A., and Thygersen, J. E., *Compt.-rend. trav. Lab. Carlsberg, Sér. chim.*, **26**, No. 14 (1948).
9 Snedecor, G. W., Statistical methods, Ames, 4th edition, 65 (1946).

[4] Unpublished observations.

METABOLIC STUDY OF THE METHYL GROUPS OF BUTTER YELLOW

By R. A. BOISSONNAS,* ROBERT A. TURNER,† AND VINCENT DU VIGNEAUD

(From the Department of Biochemistry, Cornell University Medical College, New York City)

In 1936 Kinoshita (1) reported that p-dimethylaminoazobenzene (butter yellow, DAB) caused hepatic cancer in the rat. Since that time, an extensive study has been made of the carcinogenic action of azo dyes and their degradation products. Stevenson, Dobriner, and Rhoads (2) found that DAB was demethylated during the course of its metabolism, and Miller, Miller, and Baumann (3) showed that some demethylation occurred prior to cleavage of the azo linkage. The latter workers found also that monomethylaminoazobenzene can be *methylated* in the body to form DAB. Jacobi and Baumann (4) observed that the severity of kidney hemorrhages resulting from choline deficiency could be mitigated by feeding DAB, and they suggested that DAB might act as a methyl donor. However, Baumann (5) recently attributed the protective action of DAB to causes other than transmethylation. On the other hand, György (5) has pointed out that "ceroid" pigment formation is depressed in the same direction by DAB as it is by choline and methionine.

In the present investigation a study on the fate, in the rat, of the methyl groups of DAB has been made through labeling of the methyl groups with C^{14}. For this purpose a new synthesis of DAB was devised, with monomethylaminoazobenzene as the starting point and methyl iodide and sodium in liquid ammonia as a methylating agent. The radioactivity of the butter yellow was 4.78×10^5 counts per minute per mg.

A choline-free casein diet was used in which DAB was found to be stable. The level of DAB in the diet was 0.1 per cent. A higher level was not employed on account of the toxicity of the compound. During the first 5 days the diet intake was low and the rat lost weight, but during the last 5 days the diet intake was higher and the rat gained weight steadily. A relatively low food intake and a subnormal rate of growth have been previously observed with rats fed DAB (6).

After 10 days the rat (weight, 77.4 gm.) fed radioactive DAB was

sacrificed, and choline and creatine were isolated. The data are recorded in Table I. Assuming that the total amount of choline in the rat is approximately 0.1 per cent and the creatine 0.23 per cent of the body weight (7, 8), less than 0.003 per cent of the total radioactivity ingested (0.22 mc.) appeared in the body choline and less than 0.006 per cent in the body creatine. These values are well within the experimental error of the procedure. On the normal diet employed in this work we have been unable to detect transmethylation from butter yellow.

It might be recalled that in an experiment of Keller, Rachele, and du Vigneaud (9), in which methionine labeled in the methyl group was fed for 4 days, the isolated choline possessed a radioactivity of 4.066×10^5 counts per minute per mM of methyl group. The rat ingested 3.98 mM of the methionine possessing 3.650×10^6 counts per minute per mM

TABLE I

Radioactivity of Isolated Choline and Creatinine As Compared to Ingested DAB

Compounds	Counts per min.	
	Per mg.	Per mM methyl group
Ingested radioactive DAB	4.78×10^5	5.38×10^7
Isolated choline chloroplatinate	1.6 ± 1	<165
" creatinine chloroplatinate	1.9 ± 1	<610

On the assumption of a 0.1 per cent choline content in the animal (7), these results would indicate that approximately 18 per cent of the total radioactivity ingested appeared in the choline.

Of the total radioactivity ingested as DAB, 57 per cent appeared in the expiratory carbon dioxide and 1.3 per cent in the urinary urea. The specific activities of the carbon of the expiratory carbon dioxide and of the carbon of the urea were the same, within the limits of experimental error. This is in agreement with the results of Mackenzie and du Vigneaud (10).

As this experiment proved that oxidative demethylation occurred to a large extent, we measured the rate of appearance of the radioactivity in the expiratory carbon dioxide after a single dose of radioactive DAB was administered in the diet to a rat which had previously been fed ordinary DAB. The result of this experiment is plotted in Fig. 1. The large amount of radioactive carbon dioxide expired during the first 10 hours following the feeding of radioactive DAB showed that the DAB was oxidized rapidly. It is not possible from these experiments to state whether DAB or one of its degradation products was oxidatively demethylated to the greater extent.

It would be of interest to study the oxidation of the methyl group of

DAB as well as the problem of transmethylation with DAB under dietary conditions particularly favorable for tumor formation.

<div align="center">EXPERIMENTAL</div>

Synthesis of Radioactive p-N,N-Dimethylaminoazobenzene (DAB)—In agreement with Miller and Baumann (11) we found that the direct methylation of p-aminoazobenzene with methyl iodide according to Berju (12) led to a mixture of compounds. Every attempt to improve the synthesis by using solvents and acid-neutralizing agents was unsuccessful. We devised a one-step synthesis from monomethylaminoazobenzene, using methyl iodide and sodium in liquid ammonia. 0.017 ml. (0.42 mM) of C^{14}-methanol with a radioactivity of 1.28 mc. was diluted with 0.064 ml. (1.58 mM) of ordinary methanol and converted to methyl iodide by bubbling it in a current of dry nitrogen through boiling hydriodic acid according to the procedure of Melville, Rachele, and Keller (13). The 0.12 ml. (1.88 mM) of C^{14}-methyl iodide obtained was bubbled during a period of 3 minutes in a current of dry, oxygen-free nitrogen into a solution of 422 mg. (2.0 mM) of p-N-methylaminoazobenzene (11) and 46 mg. (2.0 mM) of sodium in 75 ml. of dry liquid ammonia at −60° to −50° with continuous stirring. After 15 minutes, the initially red solution turned yellow, and an orange precipitate appeared. The liquid ammonia was evaporated, and the resulting crystalline solid was dissolved in 50 ml. of 95 per cent ethanol. 42 ml. of water were added dropwise with stirring over a period of 20 minutes. A crystalline precipitate appeared, which was kept at 0° for 15 minutes, filtered, washed 3 times with 3 ml. of 55 per cent ethanol, and dried for 1 hour at 95° *in vacuo*. The yield of DAB was 196 mg. (43.5 per cent of the theoretical amount, calculated on the basis of the methanol). Failure to obtain a higher yield can be explained by a partial ionization of the sodium salt of the amine during the synthesis. A small amount of water in the ammonia did not affect the yield appreciably.

The micro melting point was 114–116° (corrected), and after admixture with an authentic sample of DAB, it was 116.5–117°. The absorption spectrum of the compound was the same as that of authentic DAB. The radioactivity, measured after combustion of DAB and isolation of its carbon as $BaCO_3$ (13, 9) was 4.78×10^5 counts per minute per mg. of DAB, or 5.38×10^7 counts per minute per mM of methyl group.

Diet—The diet possessed the following percentage composition: DAB 0.1, vitamin-free casein (Smaco) 20.0, Osborne and Mendel salt mixture (14) 4.0, sucrose 55.9, hydrogenated vegetable oil 19.0, corn oil 1.0. It also contained vitamins in the amount included in a choline-free diet by Wilson and du Vigneaud (15). The diet was administered *ad libitum*.

At the end of the control experiment, the percentage of DAB in the diet remaining in the diet cup was measured according to the procedure of Miller and Baumann (11). No monomethylaminoazobenzene or aminoazobenzene was detected. The quantitative colorimetric analysis showed that neither demethylation nor cleavage of the azo linkage had taken place in the diet under the conditions of the experiment.

Determination of Lability of Methyl Groups of DAB—As a control of the effect of radioactivity, a 75 gm. female rat was fed for 10 days on a diet containing ordinary DAB. After a decrease in weight during the first 3 days to 72 gm., the rat started to grow again, and weighed 77.4 gm. at the end of the experiment. The average daily food intake was 4.7 gm.

The diet containing radioactive DAB was administered for 10 days to a 108 gm. female rat placed in an open circuit, metabolism apparatus (16), and the expired carbon dioxide was collected continuously in sodium hydroxide solution. The urine was collected in dilute sulfuric acid. The weight of the rat dropped to 89.4 gm. during the 5 days before the animal became accustomed to the diet. The rat weighed 93.6 gm. at the end of the experiment. The average daily food intake was 5.5 gm. during the last 5 days, as compared with 2.3 gm. during the first 5 days. The total food consumption was 38.9 gm., corresponding to 38.9 mg. of radioactive DAB, with a total count of 1.86×10^7 counts per minute.

At the end of the feeding period the rat was sacrificed. Choline was isolated from the carcass as choline chloroplatinate (17), which was recrystallized six times from water-ethanol mixtures. Creatine was isolated as creatinine potassium picrate (17), and recrystallized four times from water. It was then converted to creatinine chloroplatinate and recrystallized three times from water-ethanol mixtures. The amount of the chloroplatinates left after the recrystallizations, in which the recovery was purposely low, was 2.6 mg. in the case of the choline chloroplatinate and 2.2 in that of the creatinine chloroplatinate. The radioactivity of each chloroplatinate was determined alternately with that of the $BaCO_3$ sample obtained by combustion of DAB. The radioactivities found, corrected for background and self-absorption, are recorded in Table I. The radioactivities obtained were so low as to make further purification purposeless.

As the total amount of choline in a rat is approximately 0.1 per cent of the body weight, the total amount of radioactivity in the choline of the rat fed DAB was less than 380 counts per minute, which was less than 0.003 per cent of the radioactivity ingested.

As the total amount of creatine in a rat is about 0.2 per cent of the body weight, the total amount of radioactivity in the creatine of the rat fed DAB was less than 860 counts per minute, which was less than 0.006 per cent of the radioactivity ingested.

Measurement of Radioactivity of Expiratory Carbon Dioxide and of Urinary Urea—The expiratory carbon dioxide collected every day in 2.5 N sodium hydroxide solution was precipitated as barium carbonate and the radioactivity was determined. The percentage of the radioactivity ingested as butter yellow which was found in the expiratory carbon dioxide was 40 per cent for the first 5 days and 57.5 per cent for the 10 day period. The specific radioactivity of the expiratory carbon dioxide of the 10 day period was 28.2 counts per minute per mg. of $BaCO_3$.

The urine for the 10 day period was collected in 0.1 N sulfuric acid, and kept at 0°. It was aerated, neutralized by sodium hydroxide, buffered to pH 7, and treated with urease. The carbon dioxide was collected in sodium hydroxide solution, and precipitated as $BaCO_3$. The specific radioactivity was 27.2 counts per minute per mg. of $BaCO_3$. The total
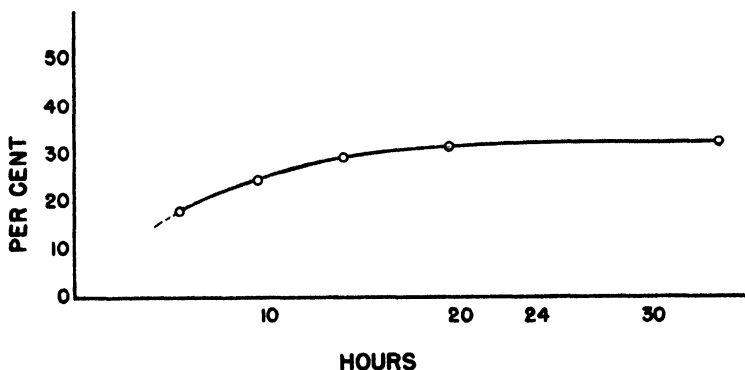
FIG. 1. Per cent of ingested radioactivity in expiratory carbon dioxide

radioactivity present in urine as urea was 1.3 per cent of the radioactivity ingested.

Rate of Appearance of Radioactivity in Expiratory CO_2—An 89 gm. female rat was fed for 4 days on the ordinary DAB diet already described. The average daily food intake was 5.7 gm., and the rat weighed 87 gm. at the end of the 4 days. Then the ordinary DAB diet was removed and replaced by radioactive DAB diet for 1 hour at the ordinary meal time of the rat. The rat ate 0.9 gm. of the radioactive diet, that is, 0.9 mg. of radioactive DAB. Then the rat was again given the ordinary DAB diet. The average food intake during the following 2 days was 6.6 gm., and the rat weighed 89.2 gm. at the end of the experiment.

The expiratory carbon dioxide was collected at intervals in sodium hydroxide solution and the radioactivity was measured after precipitation of the carbon dioxide of the sample as $BaCO_3$. The results are plotted in Fig. 1.

SUMMARY

p-N,N-Dimethylaminoazobenzene (butter yellow, DAB) containing C^{14} in the methyl groups has been synthesized by methylation of monomethylaminoazobenzene with use of methyl iodide and sodium in liquid ammonia as a methylating agent. The radioactive DAB was fed to a rat on a relatively normal casein diet for 10 days, and the choline and creatinine were then isolated from the carcass of the rat. The radioactivity of the choline and creatine was found to be negligible, showing that no appreciable transmethylation, if any, to those compounds had taken place.

The expiratory carbon dioxide contained the major fraction of the ingested radioactivity. The specific radioactivities of the carbon of the expiratory carbon dioxide and of the urinary urea were found to be the same.

The rate of appearance of the radioactivity in the expiratory carbon dioxide after the feeding of a single dose of radioactive DAB showed that oxidation of the methyl groups occurred rapidly.

BIBLIOGRAPHY

1. Kinoshita, R., *Gann*, **30**, 423 (1936); *Tr. Soc. Path. Japon.*, **27**, 665 (1937).
2. Stevenson, E. S., Dobriner, K., and Rhoads, C. P., *Cancer Res.*, **2**, 160 (1942).
3. Miller, J. A., Miller, E. C., and Baumann, C. A., *Cancer Res.*, **5**, 162 (1945).
4. Jacobi, H. P., and Baumann, C. A., *Cancer Res.*, **2**, 175 (1942).
5. Biological antioxidants, Transactions of the Second Conference, Josiah Macy, Jr., Foundation, New York, 114, Oct. 9–10 (1947).
6. Miller, J. A., Miner, D. L., Rusch, H. P., and Baumann, C. A., *Cancer Res.*, **1**, 699 (1941). White, J., *J. Nat. Cancer Inst.*, **1**, 337 (1940).
7. Jacobi, H. P., and Baumann, C. A., *J. Biol. Chem.*, **142**, 65 (1942).
8. Chanutin, A., *J. Biol. Chem.*, **89**, 765 (1930).
9. Keller, E. B., Rachele, J. R., and du Vigneaud, V., *J. Biol. Chem.*, **177**, 733 (1949).
10. Mackenzie, C. G., and du Vigneaud, V., *J. Biol. Chem.*, **172**, 353 (1948).
11. Miller, J. A., and Baumann, C. A., *Cancer Res.*, **5**, 157 (1945).
12. Berju, G., *Ber. chem. Ges.*, **17**, 1400 (1884).
13. Melville, D. B., Rachele, J. R., and Keller, E. B., *J. Biol. Chem.*, **169**, 419 (1947).
14. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
15. Wilson, J. E., and du Vigneaud, V., *Science*, **107**, 653 (1948).
16. Mackenzie, C. G., Chandler, J. P., Keller, E. B., Rachele, J. R., Cross, N., and du Vigneaud, V., *J. Biol. Chem.*, **180**, 99 (1949).
17. du Vigneaud, V., Cohn, M., Chandler, J. P., Schenck, J. R., and Simmonds, S. *J. Biol. Chem.*, **140**, 625 (1941).

A METABOLIC STUDY OF α-AMINOBUTYRIC ACID*

BY MARVIN D. ARMSTRONG AND FRANCIS BINKLEY

(*From the Laboratory for the Study of Hereditary and Metabolic Disorders and the Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City*)

A metabolic occurrence of α-aminobutyric acid was indicated by recent work of Dent, who reported the identification of α-aminobutyric acid in the urine of a patient with hepatic disease following the administration of large amounts of methionine (1). Later Fromageot and Clauser found (2) that a mixture of α-aminobutyric acid and choline was able to increase the growth of rats on a methionine-deficient diet. These experiments could be interpreted as indicating that the cleavage of the thioether cystathionine might be reductive in nature, instead of hydrolytic as had been previously suggested (3).

$$NH_2$$
$$HO—CH_2CH_2—CH—CO_2H$$
Homoserine

$$NH_2$$
$$CH_2—CH—CO_2H$$

$$+HOH$$

$$NH_2$$
$$S$$
$$CH_2—CH_2—CH—CO_2H$$

$$+2H$$

$$NH_2$$
$$+   HS—CH_2—CH—CO_2H$$
Cysteine

$$NH_2$$
$$CH_2—CH_2—CH—CO_2H$$

α-Aminobutyric acid
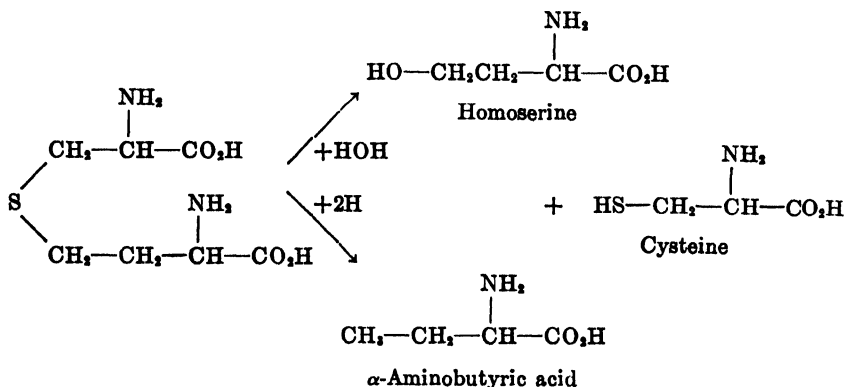
A further interest in α-aminobutyric acid was stimulated by the finding of Teas, Horowitz, and Fling (4) that homoserine could serve as a precursor of both methionine and threonine for a mutant strain of *Neurospora crassa*. This, of course, raised the possibility that the 4-carbon chain of methionine and of threonine might be the fragment necessary in the diet of the white rat for their synthesis. Examination of the literature showed that experiments had not yet been reported which could preclude the possibility that threonine might arise from α-aminobutyric acid by a

mechanism as simple as α,β-dehydrogenation followed by readdition of water to the unsaturated amino acid formed.

$$CH_3-CH_2-\underset{\underset{NH_2}{|}}{CH}-CO_2H \xrightarrow{-2H} CH_3-CH=\underset{\underset{NH_2}{|}}{C}-CO_2H \xrightarrow{HOH}$$

$$CH_3-\underset{\underset{OH}{|}}{CH}-\underset{\underset{NH_2}{|}}{CH}-CO_2H$$
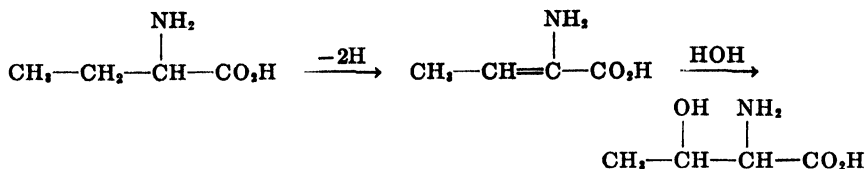
Diets lacking in methionine and in threonine, but adequate in all other respects, were administered to young white rats, and the ability of α-aminobutyric acid to substitute for each of these was tested. The results of the experiments, presented in Tables I and II, show that α-aminobutyric acid can neither substitute for threonine nor provide the 4-carbon chain for the synthesis of methionine under the conditions of the experiment.

The results of this experiment, however, do not definitely imply that in nature a synthesis of either amino acid from aminobutyric acid may not possibly occur under other conditions. It has been shown, for instance, that homoserine cannot substitute for either methionine (5) or threonine (6) in the diet of the rat, in spite of the fact that it can serve as a precursor for both amino acids for *Neurospora*.

An excellent explanation for Dent's observation of the presence of α-aminobutyric acid in the urine of a patient with hepatic injury following the administration of methionine has been offered by Carroll, Stacy, and du Vigneaud as a result of their finding that homoserine is converted to α-ketobutyric acid by liver enzymes. Thus, methionine could be converted to cystathionine, which would cleave to homoserine, and thence to α-ketobutyric acid which might undergo reductive amination to α-aminobutyric acid (7).

The results of Fromageot and Clauser appear to be somewhat more difficult to reconcile with our findings. A difference between their diet and ours was that their diet caused maintenance but did not support growth, whereas on our methionine-deficient diet the rats suffered a rapid loss in weight. The possibility thus remains that, in a diet containing a minimal amount of methionine, α-aminobutyric acid can support growth This possibility was not tested, as the purpose of the present experiment was to test the availability of α-aminobutyric acid to substitute for methionine and for threonine under conditions which would show it to be a normal, and perhaps necessary, intermediate in amino acid metabolism. A less likely explanation of the divergence of our results might lie in the fact that, since arachin was the source of the amino acids in their diet, some unknown factor could have been present, which, in the presence of α-aminobutyric acid, caused growth to occur.

EXPERIMENTAL

A litter of young white rats was used for the feeding experiment. The basal synthetic diet had the following composition: amino acid mixture (exclusive of threonine and the sulfur-containing amino acids) 20.0, sucrose 43.0, Spry 26.0, salt mixture (General Biochemicals, Inc., Salt Mixture 2) 4.0, cod liver oil 5.0, and agar 2.0 parts, respectively. The amino acid

TABLE I

Results of Growth Experiments on Threonine-Deficient Diet

Rat No	Days	Average daily food intake	Average daily weight change	Supplement to basal diet
		gm.	*gm.*	*per cent*
18 ♀	1- 8	5 4	+1.6	0.8 DL-methionine + 1.4 DL-threonine
	9–20	5.5	+2.4	0.8 " + 1.4 "
17 ♀	1- 8	4.6	+0.9	0.8 " + 1.4 "
	9–20	2 5	−0.9	1.4 " + 1.5 DL-aminobutyric acid
	21–30	3.1	−0.8	1.4 DL-methionine + 1.5 DL-aminobutyric acid
22 ♀	1- 8	2.9	−1.8	1.4 DL-methionine
	9–20	1.9	−0.5	1.4 " + 1.5 DL-aminobutyric acid
	21–30	2.2	−0.7	1.4 DL-methionine + 1.5 DL-aminobutyric acid
23 ♀	1- 8	2.3	−1.6	1.4 DL-methionine
	9–20	1.8	−0.7	1.4 " + 1.5 DL-aminobutyric acid
	21–23	2.3	−1.3	1.4 DL-methionine + 100 mg. aminobutyric acid per day
	24–30	2.6	+1.3	1.4 DL-methionine + 60 mg. DL-threonine per day
24 ♂	1- 8	2.5	−1.8	1.4 DL-methionine
	9–20	2.0	−0.4	1.4 "
	21–30	1.7	−0.5	1.4 "

mixture was composed of crystalline amino acids and was essentially that reported by Rose and Rice (8), with the exception that serine, proline, and hydroxyproline were omitted and the relative amounts of the remaining amino acids were altered slightly. The water-soluble vitamins were given as a solution, each rat receiving daily in two portions 1.0 cc. of a solution which contained 20 γ of thiamine chloride, 20 γ of riboflavin, 20 γ of nicotinic acid, 20 γ of pyridoxine hydrochloride, 100 γ of calcium d-pantothenate, and 25.0 mg. of choline chloride. DL-Methionine, L-cystine, and DL-α-aminobutyric acid were incorporated in the

diets in the amounts shown in Tables I and II and an equal weight of sucrose was omitted from the diet. Food and water were allowed *ad libitum,* the food consumption was measured daily and the animals were weighed at intervals.

The results of the experiment are tabulated in Tables I and II. It is apparent that in the case of both experiments the animals receiving

TABLE II

Results of Growth Experiments on Methionine-Deficient Diet

Rat No.	Days	Average daily food intake	Average daily weight change	Supplement to basal diet
		gm.	*gm.*	*per cent*
16 ♂	1– 8	5.3	+2.0	0.8 DL-methionine + 1.4 DL-threonine
	9–20	3.0	−0.4	1.5 DL-aminobutyric acid + 1.0 L-cystine + 1.4 DL-threonine
	21–30	3.4	−0.9	1.5 DL-aminobutyric acid + 1.0 L-cystine + 1.4 DL-threonine
18 ♀	1– 8	5.4	+1.6	0.8 DL-methionine + 1.4 DL-threonine
	9–20	5.5	+2.4	0.8 " + 1.4 "
	21–30	3.0	−1.8	1.0 L-cystine + 1.4 DL-threonine + 100 mg. DL-aminobutyric acid per day
19 ♀	1– 8	3.3	−1.3	1.0 L-cystine + 1.4 DL-threonine
	9–20	3.0	−0.3	1.0 " + 1.4 " + 1.5 DL-aminobutyric acid
	21–30	3.0	−0.4	1.0 L-cystine + 1.4 DL-threonine + 100 mg. DL-aminobutyric acid per day
20 ♀	1– 8	3.1	−1.5	1.0 L-cystine + 1.4 DL-threonine
	9–20	2.8	+0.2	1.0 " + 1.4 " + 1.5 DL-aminobutyric acid
	21–30	2.5	−0.5	1.0 L-cystine + 1.4 DL-threonine + 1.5 DL-aminobutyric acid
21 ♀	1– 8	2.9	−1.1	1.0 L-cystine + 1.4 DL-threonine
	9–20	2.3	−0.25	1.0 " + 1.4 "
	21–30	2.2	−0.2	1.0 " + 1.4 "

the aminobutyric acid supplements declined at about the same rate as the animals receiving no supplement.

SUMMARY

It has been found that DL-α-aminobutyric acid cannot substitute as a dietary precursor in the white rat for the 4-carbon basic fragment either of methionine or of threonine in diets containing pure amino acids instead of protein as a source of nitrogen.

BIBLIOGRAPHY

1. Dent, C. E., *Biochem. J.*, **40**, p. xliv (1946); *Science*, **105**, 335 (1947).
2. Fromageot, C., and Clauser, H., *Biochim. et biophys. acta*, **1**, 449 (1947).
3. Binkley, F., *J. Biol. Chem.*, **155**, 39 (1944).
4. Teas, H. J., Horowitz, N. H., and Fling, M., *J. Biol. Chem.*, **172**, 651 (1948).
5. Armstrong, M. D., and Binkley, F., *J. Biol. Chem.*, **174**, 889 (1948).
6. McCoy, R. H., Meyer, C. E., and Rose, W. C., *J. Biol. Chem.*, **112**, 283 (1935–36).
7. Carroll, W. R., Stacy, G. W., and du Vigneaud, V., *J. Biol. Chem.*, **180**, 375 (1949).
8. Rose, W. C., and Rice, E. E., *J. Biol. Chem.*, **130**, 305 (1939).

A FLUOROMETRIC METHOD FOR THE ESTIMATION OF TRYPTOPHAN*

By MALCOLM GORDON† AND HERSCHEL K. MITCHELL

(From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena)

The various colorimetric methods now employed for the estimation of tryptophan are not specific for tryptophan but give colored products with many compounds containing the indole nucleus, including indole itself. In order to facilitate studies of the enzymatic synthesis of tryptophan from indole and serine by extracts of *Neurospora* (1), a search for a rapid, quantitative method for the estimation of tryptophan in the presence of indole was undertaken. Tauber (2) reported that tryptophan gives a green fluorescence when treated with 70 to 72 per cent perchloric acid at room temperature. Modification of this procedure has led to a method for the rapid estimation of tryptophan, without preliminary extraction of indole, in hydrolyzed or unhydrolyzed tryptophan-containing materials.

EXPERIMENTAL

Apparatus—Intensity of fluorescence is measured with a Coleman electronic photofluorometer, model 12B. The light entering from the mercury vapor lamp is caused to pass through a PC-3 filter and the resulting fluorescence through a PC-10 filter. A ¾ in. round test-tube of the type ordinarily used with this instrument is fitted with a leucite ring bored to support a 22 × 100 mm. test-tube inside the larger tube. Test solutions are placed in the inner tube. A volume of 2.5 ml. is sufficient to allow the entering light to focus in the test solution. Under these conditions readings are reproducible to within ±2 scale divisions.

Reagents—

1. 70 to 72 per cent perchloric acid (Merck reagent).

2. Aqueous indole. Dissolve 117 mg. of indole (Eastman) in 100 ml. of distilled water.

3. Aqueous γ-(indole-3)-n-butyric acid. Dissolve 10 mg. of γ-(indole-3)-n-butyric acid (Eastman) in 100 ml. of distilled water.

4. 10 per cent acid-hydrolyzed casein (Nutritional Biochemicals Corporation).

5. Phosphate buffer, pH 7.5 to 8.0. Dissolve 14.7 gm. of disodium hydrogen phosphate (Merck reagent) in distilled water and make up to a liter. Adjust pH with phosphoric acid.

6. Supplement. Mix 1 ml. of indole solution, 1 ml. of γ-(indole-3)-n-butyric acid solution, and 1 ml. of 10 per cent acid-hydrolyzed casein. Dilute to 10 ml. with phosphate buffer. The supplement should be made up fresh weekly and kept in a refrigerator when not in use.

Procedure for Assay—In order to obtain results which have quantitative significance it is necessary to stabilize the fluorescence. Such stabilization can be effected by the use of the supplement described above. The sample to be tested is taken up in the phosphate buffer and 0.4 ml.

TABLE I

Fluorescence Obtained by Treating Certain Indole Derivatives with 70 to 72 Per Cent Perchloric Acid

Each tube contains 0.05 μM of the compound.

Compound	Scale reading	Compound	Scale readi
Indole*	2	β-(Indole-3)-n-propionic acid	70
Indole-2-carboxylic acid	1	γ-(Indole-3)-n-butyric acid	35
Indole-3-carboxylic acid	2	Tryptophan*	10
3-Methylindole	38		
Indole-3-acetic acid	55		

*The photofluorometer is adjusted to a zero scale reading with a mixture of 0.5 ml. of distilled water and 2 ml. of 70 to 72 per cent perchloric acid. The sensitivity is so adjusted that the tryptophan tube reads 100.

is added to 0.1 ml. of the supplement solution. 2 ml. of perchloric acid are added rapidly at room temperature, the solution is well mixed, warmed for 1 hour at 40°, and allowed to cool to room temperature. A series of tubes prepared in the same manner containing 0, 2, 4, 6, and 8 γ of tryptophan is used to adjust the photofluorometer. The instrument is adjusted to a zero scale reading with the blank tube and the sensitivity is adjusted with the 8 γ tube to a scale reading of 100. The intermediate tubes will read approximately 25, 50, and 75, respectively. The unknown solutions are read with this setting of the instrument. Occasional readjustments of the zero and 100 readings are desirable during the course of a large number of determinations. Since the intensity of fluorescence slowly diminishes, it is necessary to adjust the instrument with a control series prepared at the same time as the unknown solutions. The linear relationship between the intensity of fluorescence and the concentration of tryptophan persists for about 24 hours.

Influence of Various Materials on Relative Stability of Fluorescence—The relative stability of fluorescence is a sensitive function of several materials which do not contain tryptophan. High concentrations of many inorganic

TABLE II

Tryptophan Found in Various Materials (in Per Cent Tryptophan)

Material	Unhydrolyzed*	Hydrolyzed	Literature values
Albumin egg scales†	2.04	1.88	
	1.98	1.96	
	2.04		
Crystalline bovine serum albumin‡	0.72	0.68	0.57 (4)
	0.76	0.70	0.65 (5)
	0.69	0.65	0.68 (5)
Casein§	1.40	1.33	1.32 (4)
	1.32	1.27	1.38 (5)
	1.38	1.24	1.54 (6)
Edestin‖	1.44	1.30	1.3 (7)
	1.46	1.29	1.3 (8)
	1.38	1.32	1.2 (4)
			1.4 (10)
Gelatin¶	0.016	0.010	0.0 (11)
	0.021	0.012	0.0 (9)
	0.017		0.1 (12)
Gluten**	0.84	0.75	0.91 (4)
	0.86	0.81	0.93 (6)
	0.94		1.2 (13)
Zein††	0.22	0.21	0.2 (14)
	0.20	0.19	0.2 (9)
			0.0 (12)
Hot water *Neurospora* extract (autolyzed)	0.34	0.30	
	0.32	0.29	
Acid-hydrolyzed casein + 2% added tryptophan	2.00	1.89	
	2.08	1.84	
	2.04	1.90	

* Separate determinations on the same sample.
† Albumin egg scales, J. T. Baker Chemical Company.
‡ Crystallined bovine serum albumin, the Armour Laboratories, G 4502 plasma.
§ Casein, vitamin-free S. M. A. Corporation batch No. 12537.
‖ Edestin, laboratory sample of unknown origin.
¶ Gelatin, Difco batch No. 326614.
** Gluten, Braun, Knecht, Heimann Company.
†† Zein (corn), Pfanstiehl Chemical Company, batch No. 536.

ions, concentrations of organic materials above 10 mg. per tube, prolonged heating or exposure to direct sunlight or ultraviolet light, and either too high or too low a concentration of hydrogen ion cause uneven fading of fluorescence. The Na_2HPO_4 adjusts for this last effect, except in strongly

alkaline or acidic solutions.   In such cases the sample should be neutralized before being taken up in the buffer solution.

Occasional samples of perchloric acid, when mixed with tryptophan by the described procedure, will not produce the substances which fluoresce under ultraviolet light.   It seems probable that these samples are contaminated with one or more agents which inhibit fluorescence.
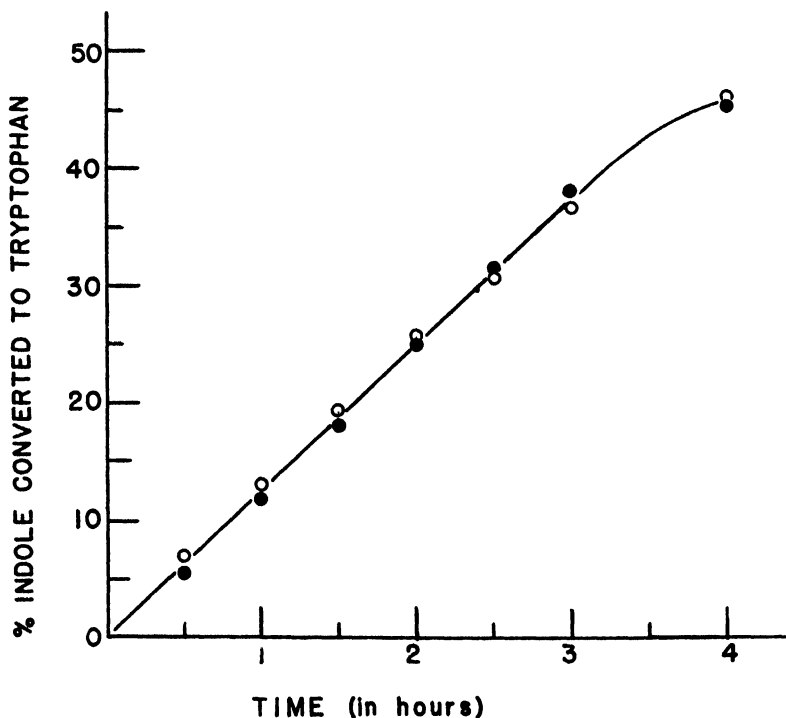
FIG. 1. Per cent conversion of indole to tryptophan by extracts of *Neurospora*. O, calculated from tryptophan found by perchloric acid method; ●, calculated from indole found by xanthydrol method of Fearon.

Specificity of Test—Table I gives data comparing the fluorescence obtained when 0.05 μM of several compounds containing an indole nucleus is treated with 70 to 72 per cent perchloric acid.   Tests for tryptophan based upon its reaction with aldehydes in the presence of mineral acids are thought to be characteristic of indole and indole derivatives containing a hydrogen-substituted 2-carbon atom.   In contrast, the fluorescence obtained in this test appears to be a function of both a hydrogen-substituted 2-carbon atom and a side chain in the 3 position of the indole nucleus.

Estimation of Tryptophan in Various Organic Materials—Table II gives

tryptophan values obtained by the above procedure for various substances both before and after hydrolysis for 1 hour with 2 N NaOH. All samples were dried *in vacuo* for 4 hours at 65° over P_2O_5. Literature values for tryptophan in similar materials are listed for comparison.

Fig. 1 is a plot of the increase of tryptophan concentration found by this method when a crude dialyzed *Neurospora* extract is treated with pyridoxal phosphate, indole, and serine, and incubated at 35° at pH 7.8. On the same graph is plotted the percentage conversion of indole to tryptophan obtained by measuring the decrease in indole concentration by the method of Fearon (3).

DISCUSSION

The nature of the reactions involved in the method herein described is unknown. Therefore, justification of the use of the recommended mixtures of amino acids, indole, and γ-(indole-3)-n-butyric acid to obtain relative stability of fluorescence must be a strictly empirical one. However, other workers have recommended the use of amino acid mixtures to stabilize tryptophan in highly acidic solutions (4, 15, 16). Graham *et al.* (4) suggest the use of 35 mg. of gelatin in order to stabilize the color obtained when tryptophan is estimated by the Bates (17) procedure. Since the samples of gelatin tested during this investigation contained detectable traces of tryptophan, attempts were made to obtain relative stability of fluorescence by use of acid-hydrolyzed casein. All experiments with this in view were negative and the addition of an indole-containing compound with a side chain in the 3 position was required before adequate stability was obtained. The test was standardized with γ-(indole-3)-n-butyric acid because of the low fluorescence produced by it in this procedure. Although the test could no doubt be modified by using small amounts of tryptophan itself as the stabilizing indole component, it seems less ambiguous to use a material which is unknown in biological systems.

Readings on solutions containing 1 to 8 γ of tryptophan can be reproduced to within ±2 scale divisions. However, with scale readings below 50, this represents an error of at least 4 per cent, depending upon where the reading falls on the scale. If the samples to be tested are chosen so that they contain 6 to 8 γ of tryptophan, determinations can be repeated with an error of not more than ±5 per cent.

Tauber (18) has reported that small quantities of dichromate enhance the fluorescence obtained in his qualitative test. With the quantities of tryptophan used in this work, dichromate was found to have a deleterious effect on the stability of the fluorescence.

SUMMARY

A new method for the estimation of tryptophan has been described. Tryptophan can be quantitatively determined in unhydrolyzed proteins. Determinations are reproducible approximately to within 5 per cent.

BIBLIOGRAPHY

1. Umbreit, W. W., Wood, W. A., and Gunsalus, I. C., *J. Biol. Chem.*, **165**, 731 (1946).
2. Tauber, H., *J. Am. Chem. Soc.*, **70**, 2615 (1948).
3. Fearon, W. R., *Analyst*, **69**, 122 (1944).
4. Graham, C. E., Smith, E. P., Hier, S. W., and Klein, D., *J. Biol. Chem.*, **168**, 711 (1947).
5. Hauschildt, J. D., Isaacs, T. L., and Wallace, W. B., *J. Biol. Chem.*, **167**, 331 (1947).
6. Greenhut, I. T., Schweigert, B. S., and Elvehjem, C. A., *J. Biol. Chem.*, **165**, 325 (1946).
7. Shaw, J. L. D., and McFarlane, W. D., *J. Biol. Chem.*, **132**, 387 (1940).
8. Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, **83**, 103 (1929).
9. Folin, O., and Looney, J. M., *J. Biol. Chem.*, **51**, 421 (1922).
10. Kiesel, A., and Kusmin, S., *Z. physiol. Chem.*, **238**, 145 (1936).
11. Albanese, A. A., and Frankston, J. E., *J. Biol. Chem.*, **144**, 563 (1942).
12. May, C. E., and Rose, E. R., *J. Biol. Chem.*, **54**, 213 (1922).
13. Padoa, M. L., *Ann. chim. appl.*, **21**, 544 (1938); *Chem. Abstr.*, **32**, 6297 (1938).
14. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).
15. Sullivan, M. X., Milone, H. S., and Everitt, E. L., *J. Biol. Chem.*, **125**, 471 (1938).
16. Doty, D. M., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 169 (1941).
17. Bates, R. W., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **119**, p. vii (1937).
18. Tauber, H., *J. Biol. Chem.*, **177**, 337 (1949).

SEPARATION OF ENZYMES ON THE FILTER PAPER CHROMATOPILE*

By HERSCHEL K. MITCHELL, MALCOLM GORDON,† AND FRANCIS A. HASKINS

(*From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena*)

A recent report from this laboratory described a method for isolation of low molecular weight compounds by chromatography on a pile of filter paper disks (1). Preliminary experiments have now been completed on the application of this method to the separation of enzymes. It was expected that a mixture of enzymes could be placed near the top of a filter paper pile and fractionated by imposing a gradient (*i.e.* pH, organic solvent, or salt concentration) on the column, thus taking advantage of a continuous process. Following the observation of Tiselius (2) it also seemed probable that enzyme separations could be obtained on the filter paper pile by salting-out adsorption and adsorption displacement.

EXPERIMENTAL

Concentration Gradients—Although some separation of adenosine deaminase was obtained from amylase in taka-diastase by means of a pH gradient in 0.1 M phosphate buffer, more promising results were derived from the use of an $(NH_4)_2SO_4$ concentration gradient. The following experiment is illustrative. Taka-diastase (300 mg. of 525 per cent with respect to amylase activity; Parke, Davis and Company) was dissolved in 20 ml. of water and taken up on twenty sheets of 9 cm. Whatman No. 1 filter paper. After drying at room temperature, the twenty disks were incorporated at forty disks from the top of a 500 sheet pile (1). The entire apparatus and solvents were equilibrated at 10°. The solvent distributor and syphon were filled with 60 per cent saturated $(NH_4)_2SO_4$ solution (pH 6.5)[1] and the syphon tube placed in a beaker containing 100 ml. of the same $(NH_4)_2SO_4$ solution. The solvent surface was placed level with the top of the column. Distilled water was added from a drop-

[1] All $(NH_4)_2SO_4$ concentrations are given for 10°. All pH values were determined without dilution with a Beckman pH meter.

ping funnel at such a rate as to maintain the solvent level in the beaker. A thorough mixing was obtained by a stirring motor. After 8 hours the column had taken up 275 ml. of solvent in 450 paper disks. The column was removed from the clamp and alternate sections of five disks each were removed and cut into 0.5 × 4 cm. strips for measurement of activity on five sheets at a time. Activities of amylase (3), adenylic acid phosphatase (3), and adenosine deaminase (4) were measured by methods
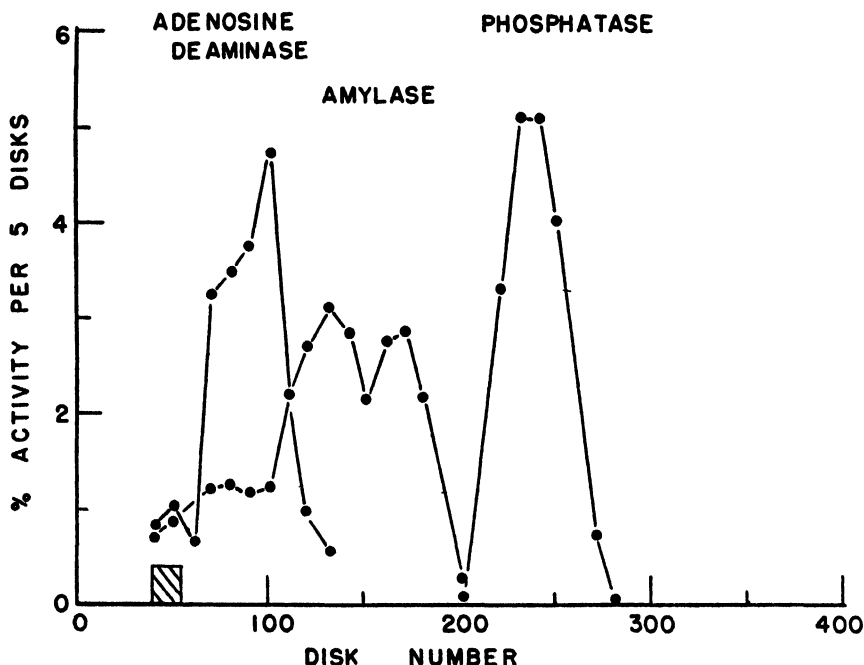
FIG. 1. Separation of three enzymes on the filter paper chromatopile with an (NH₄)₂SO₄ gradient at pH 6.5. Salt concentration in per cent saturation at 10°; Disk 30, 14 per cent; Disk 120, 20 per cent; Disk 202, 40 per cent; Disk 280, 50 per cent.

previously described. Paper strips from each section were dropped directly into the substrate solutions without extraction of the enzymes from the paper or removal of the (NH₄)₂SO₄. Controls of unfractionated taka-diastase were carried out in the same fashion. The results of one experiment of this kind are summarized in Fig. 1. The figures for (NH₄)₂SO₄ concentration are calculated as per cent saturation at 10° based on the average volume of solvent per disk (0.61 ml.) in the pile. These values were determined by extraction of sheets in hot water, followed by a colorimetric determination of NH₃ by nesslerization. The calculated total

recoveries of enzyme activities were adenosine deaminase 85 per cent, amylase 120 per cent, and phosphatase 65 per cent. These values are based on controls with the original enzyme solutions absorbed on paper. Similar results were obtained by extraction of the enzymes from the paper before measurement of the activities, but this procedure was cumbersome and found to be unnecessary.

Although extensive investigations have not been carried out to determine the effects of various salts, the effect of a pH gradient in a constant
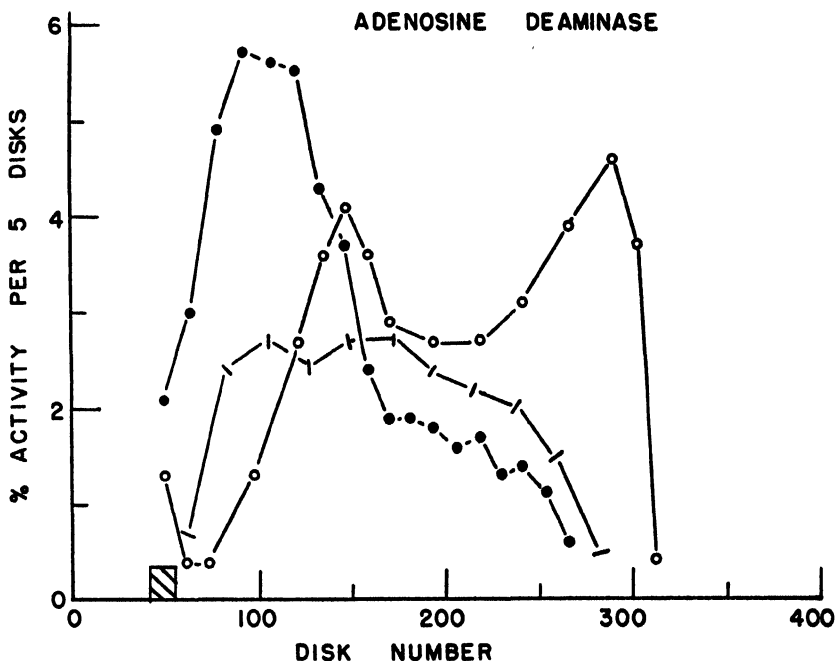
FIG. 2. Movement of adenosine deaminase in the chromatopile at different $(NH_4)_2SO_4$ concentrations and at pH 6.5. ●, 20 per cent saturation; O, 31 per cent saturation; dash line, 45 per cent saturation.

salt concentration has been considered to a limited extent. These experiments were prompted by the finding that, in second runs with samples of twenty disks taken from activity peaks of amylase and adenosine deaminase from a preliminary pile fractionation, both enzymes failed to move significantly. The second runs were carried out at pH 6.5 in $(NH_4)_2SO_4$ concentrations as low as 10 per cent saturation. A pH gradient was therefore produced in 30 per cent saturated $(NH_4)_2SO_4$ solution by dropwise addition of the salt solution, 0.25 M in NH_4OH, to salt solution 0.1 M in phosphate buffer in the same fashion previously described for producing a salt gradi-

ent. Amylase activity was found to move satisfactorily in second runs above pH 7.3 but not in the range 4.9 to 7.3. Adenosine deaminase moved significantly at the highest pH attained (7.7) but not at lower pH values. Specific details on the simultaneous variation of salt concentration and pH will be considered subsequently. It is already clear that such variations will provide another means of obtaining protein separations.
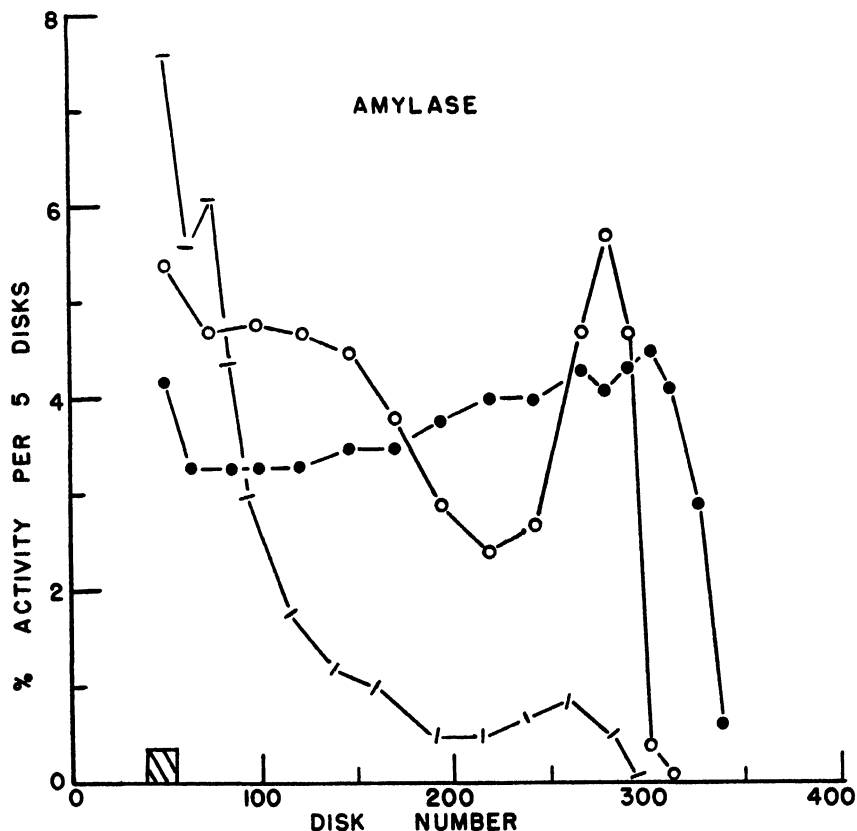
FIG. 3. Movement of amylase in the chromatopile at different $(NH_4)_2SO_4$ concentrations and at pH 6.5. ●, 20 per cent saturation; O, 31 per cent saturation; dash line, 45 per cent saturation.

Constant Salt Concentrations—Amylase and adenosine deaminase activities were determined in four 350 sheet piles with 20, 30, 45, and 60 per cent saturated $(NH_4)_2SO_4$ respectively, as solvents. The experiments were carried out essentially as described above for the gradient pile. The results are summarized in Figs. 2 and 3. Data are not shown for the 60 per cent saturation level of $(NH_4)_2SO_4$, since both enzymes failed to move

significantly at this concentration level. It should be noted, however, that neither the adenosine deaminase nor the amylase of the crude mixture is precipitated from solution by 60 per cent saturation of $(NH_4)_2SO_4$.

DISCUSSION

It is evident from the experiments described that the chromatopile provides a convenient column for the fractionation of enzymes. Although the initial experiments were designed to separate enzymes simply on the basis of a continuous process involving solubility with a solvent gradient, it is clear that adsorption of the proteins on the paper plays a significant rôle and a gradient is not necessarily essential. In agreement with the report of Tiselius (2), the enzymes are strongly adsorbed by the paper at salt concentrations far below those required for precipitation. In addition, the movement of an enzyme in the pile is markedly influenced by the presence of other proteins. This may be interpreted in terms of displacement adsorption, as previously demonstrated (2). However, by appropriate adjustment of pH, displacement adsorption may become less significant, as indicated by repeated fractionation experiments. The data of Fig. 2 for adenosine deaminase show that the rate of movement of the enzyme goes through a maximum as the $(NH_4)_2SO_4$ concentration is increased. Although the picture is complicated by the presence of many proteins, it seems probable that this phenomenon is due in part to a simultaneous effect of the salt on the adsorbability of the protein and on the properties of the paper as an adsorbent.

Throughout the experiments that have been carried out there has been consistent evidence for a number of activity peaks with both amylase and the more specific enzyme adenosine deaminase (Figs. 2 and 3). Although these results suggest multiple molecular species for both enzymes, such a conclusion cannot be justified on the basis of the present experimental evidence. This question is being investigated further.

SUMMARY

1. It has been established that considerable enzyme resolution can be attained by use of the filter paper chromatopile.

2. Variations in salt concentrations and pH are shown to have a great influence on the movement of at least two enzymes in the pile. These variations with the many obvious ones that are yet to be investigated may provide some new criteria for protein homogeneity.

3. Sufficient quantities of proteins can be fractionated on the chromatopile to provide a means of isolation.

BIBLIOGRAPHY

1. Mitchell, H. K., and Haskins, F. A., *Science*, in press.
2. Tiselius, A., *Chem. and Eng. News*, **27**, 1041 (1949).
3. Sumner, J. B., and Somers, G. F., Chemistry and methods of enzymes, New York (1946).
4. Mitchell, H. K., and McElroy, W. D., *Arch. Biochem.*, **10**, 343 (1946).

THE AMINO ACID COMPOSITION OF PORK AND LAMB CUTS

By B. S. SCHWEIGERT, BARBARA TAIT GUTHNECK, H. R. KRAYBILL,
AND D. A. GREENWOOD*

(*From the American Meat Institute Foundation, University of Chicago, Chicago*)

The development of accurate, sensitive microbiological methods has permitted the acquisition of data on the amino acid content of foods and purified proteins as well as information on the amino acids in body fluids and on the metabolism of the amino acids by the test organisms. These advances have been summarized in several reviews (1–4). The data obtained thus far on meats indicate that the amino acid composition of the protein of the lean tissues of pork, beef, and lamb cuts is very similar, although some differences in the protein of muscle tissues and organ meats have been observed in the case of histidine and lysine (5, 6).

The present study was undertaken to obtain further data on the amino acid content of different pork and lamb cuts, to evaluate different methods of analysis where indicated, and to obtain further information on the stability of the amino acids during cooking of the meats. The data obtained for leucine, valine, isoleucine, phenylalanine, threonine, histidine, lysine, arginine, methionine, and tryptophan are presented in this paper.

EXPERIMENTAL

Paired pork and lamb cuts were obtained and one cut from each pair was cooked by recommended methods (7), while the remaining cut was retained for analysis of the uncooked meat. Proximate analysis data were obtained on the fresh and cooked cuts. The samples were then partially dehydrated and defatted, finely ground, and retained for the amino acid studies. All amino acid analyses were made on the dehydrated and defatted samples. From these data and the proximate analyses, the amino acid content of the crude protein (N × 6.25) and of the fresh cuts was calculated.

The amino acids were liberated from the samples by acid autoclaving (0.5 gm. of sample in 25 ml. of 3 N HCl for 16 hours at 15 pounds pressure). The amino acid determinations were made on suitable neutralized aliquots. All analyses were carried out on duplicate hydrolysates and excellent agreement in the values was obtained for the two hydrolysates. Leucine, valine, isoleucine, and phenylalanine were determined

* Present address, Department of Chemistry, Utah State Agricultural College, Logan, Utah.

with *Lactobacillus arabinosus* as the test organism, arginine, histidine, and threonine with *Streptococcus faecalis* R., and lysine, methionine, histidine, and phenylalanine with *Leuconostoc mesenteroides* P-60 as the test organism.

<div align="center">

TABLE I

Composition of Media

</div>

Constituent*	Amount per 100 ml.	Constituent†	Amount per 100 ml.
			mg.
Glucose	4 gm.	Glutamic acid	80
Sodium acetate	4 "	Asparagine	80
Adenine	2 mg.	Lysine	40
Guanine	2 "	Cystine	40
Uracil	2 "	Leucine	20
Xanthine	2 "	Threonine	20
Salts A	1 ml.	Valine	20
" C (Henderson-Snell	1 "	Isoleucine	20
(12))		Alanine	20
Thiamine	100 γ	Methionine	20
Riboflavin	100 "	Arginine	20
Nicotinic acid	200 "	Histidine	20
Ca pantothenate	100 "	Tryptophan	20
Pyridoxine	100 "	Tyrosine	20
Pyridoxal	100 "	Serine	10
p-Aminobenzoic acid	25 "	Phenylalanine	10
Biotin	2 "	Proline	10
Pteroylglutamic acid	2 "	Glycine	20

* The amounts as given for 100 ml. of double strength medium are those added per 100 tubes, with a final volume of 2 ml. of sample, water, and medium per tube. For *S. faecalis* R assays the sodium acetate and Salts A are replaced by 5 gm. of sodium citrate and 1 gm. of K_2HPO_4 per 100 ml. Salts A is composed of 100 mg. of KH_2PO_4 and 100 mg. of K_2HPO_4 per ml.

† The amounts indicated are for the L isomer; where the DL isomer is used, twice the amounts indicated are added. For the methionine assays the amino acids were replaced by 1500 mg. of H_2O_2-treated peptone (13), cystine, tryptophan, and tyrosine at the amounts indicated per 100 ml.

It is recognized that, if any racemization of the amino acids occurred during hydrolysis, the analytical values would be low, since the D isomers of these amino acids are inactive or essentially inactive for the test microorganisms.

The methods of analysis used were essentially those described in earlier work (8–10); however, the composition of the media has been changed somewhat from that used in the earlier work. Details are given in Table I. All assays were carried out with a final volume of 2.0 ml. and the acid production after incubating for 72 hours was determined titrimetrically.

Tryptophan was determined by the Bates method essentially as described by Graham and associates (11).

RESULTS AND DISCUSSION

The assay results obtained with the present media were very satisfactory. Tests to confirm the validity of the results obtained for histidine and phenylalanine revealed that excellent agreement in the assay values was obtained with the use of two different test organisms when applied to both pork and lamb samples (Table II).

TABLE II

Comparison of Histidine and Phenylalanine Values Obtained with Use of Different Test Organisms

The values are given in the per cent of the partially dried and defatted samples.

Sample; description	Histidine		Phenylalanine	
	S. faecalis R	*L. mesenteroides*	*L. arabinosus*	*L. mesenteroides*
Lamb, rib chop	2.24	2.32	3.37	3.31
" shoulder	2.06	2.13	2.91	2.95
" leg	2.04	2.13	3.26	3.01
" breast	2.24	2.10	3.17	3.23
Pork, steak	2.59	2.37	3.35	3.26
" loin	2.86	2.92	3.32	3.48
" spareribs	2.46	2.46	3.62	3.66

Other studies were conducted to compare the methionine values obtained by the microbiological method with those obtained by a chemical method (14) and also to compare different media with *L. mesenteroides* and *S. faecalis* R. The results obtained with the microbiological method were in good agreement with those obtained by the chemical method for samples varying over a wide range of potency (Table III). The addition of yeast extract (Difco) to the medium did not influence the assay values for methionine (Table III) for samples that were relatively low in accessory factors (casein and soy bean meal) or for samples that were rich in growth stimulants (lung and liver meal and pork muscle). Other tests with the purified amino acid medium described in Table I either with or without the addition of yeast extract did not reveal any consistent deviations from the methionine values indicated with either *S. faecalis* R or *L. mesenteroides* as the test organism.

The amounts of leucine, valine, isoleucine, threonine, phenylalanine, arginine, histidine, lysine, methionine, and tryptophan in the crude protein of pork and lamb samples are presented in Tables IV and V. The percentage of protein in the fresh and cooked samples is also presented

Table III

Comparison of Chemical and Microbiological Determination of Methionine*

The values are given in per cent methionine in the crude protein.

Sample; description	Chemical method	Microbiological method†	
		Medium A	Medium B
Fish-meal	2.90	2.85	
Gelatin	0.90	0.80	
Soy bean meal, No. 1	1.14	1.15	
" " " " 2		1.06	1.12
Casein, No. 1	2.66	2.65	
" " 2		2.77	2.84
Pork muscle		2.55	2.43
Lung and liver meal		1.64	1.65

* Part of this study was carried out at the Agricultural and Mechanical College of Texas (B. S. Schweigert, Patricia Sparks, and Frances Panzer).

† *L. mesenteroides* was used as the test organism. Medium A is the peptone medium described in Table I and Medium B is the same, with the addition of 0.1 mg. of yeast extract per tube as a source of additional growth factors.

Table IV

Amino Acid Content of Fresh and Cooked Pork*

Sample; description	Protein	Ether extract	Moisture	Leucine	Valine	Isoleucine	Threonine	Phenylalanine	Arginine	Histidine	Lysine	Methionine	Tryptophan
Fresh samples													
Rib chop	16.31	27.8	54.5	7.28	4.90	4.80	5.06	3.71	6.12	3.15	7.85	2.43	1.33
Loin "	16.53	26.9	55.7	7.76	4.98	4.78	5.21	3.70	5.71	3.56	8.06	2.49	1.40
Steak	12.62	40.1	47.6	7.16	4.81	4.82	4.80	3.86	5.85	2.72	7.98	2.58	1.33
Loin	16.36	26.1	56.8	7.26	4.82	4.64	5.13	4.00	6.12	3.53	7.76	2.57	1.22
Spareribs	14.20	38.6	47.7	7.58	5.00	5.02	5.37	4.47	6.32	2.88	8.02	2.67	1.34
Shoulder	14.84	32.9	51.9	7.11	4.94	4.71	5.08	4.16	5.87	3.51	7.40	2.42	1.39
Average...........				7.36	4.91	4.80	5.11	3.98	6.00	3.23	7.85	2.53	1.33
Cooked samples													
Rib chop	21.00	42.6	36.1	7.67	5.22	4.87	5.28	4.28	7.30	3.37	7.90	2.29	1.36
Loin "	23.19	41.1	36.2	7.68	5.06	5.25	4.98	4.24	6.77	3.45	7.37	2.48	1.45
Steak	17.98	48.3	32.4	8.06	5.01	4.94	5.23	4.60	6.46	2.91	7.91	2.61	1.43
Loin roast	19.68	40.5	39.0	7.96	5.00	4.80	4.98	4.24	6.48	3.37	8.20	2.45	1.37
Spareribs	23.22	41.6	34.3	7.01	4.92	4.82	5.06	4.17	6.68	2.80	7.22	2.42	1.36
Shoulder	22.96	33.2	43.3	7.75	5.00	5.18	5.29	4.22	6.52	3.45	7.52	2.55	1.47
Average...........				7.69	5.03	4.98	5.14	4.29	6.70	3.23	7.69	2.47	1.40

* The values for protein, ether extract, and moisture are expressed as the percentage in the moist meat and the values for the amino acids are expressed as the percentage in the crude protein (N × 6.25).

and the amount of each amino acid in these samples can be calculated by multiplying the percentage of the amino acid in the protein by the protein percentage and dividing by 100. The amount of the amino acids in the different cuts of pork and lamb is quite uniform, with the exception of histidine. Although the histidine values for lamb are in good agreement for different cuts, they are lower than those for pork. The amount

TABLE V

*Amino Acid Content of Fresh and Cooked Lamb**

mple; description	Pro-tein	Ether extract	Moisture	Leucine	Valine	Isoleucine	Threonine	Phenylalanine	Arginine	Histidine	Lysine	Methionine	Tryptophan
					Fresh samples								
Rib chop	14.45	38.3	46.5	6.82	5.02	4.24	4.35	3.83	6.52	2.43	7.04	2.21	1.22
Loin "	14.47	33.8	51.0	6.97	5.16	4.82	4.78	3.83	6.95	2.59	7.88	2.48	1.30
Shoulder	18.33	11.8	69.3	7.67	5.12	4.64	4.98	3.78	6.97	2.53	7.97	2.61	1.45
Leg	15.23	32.2	53.4	7.15	4.74	4.60	4.79	3.72	6.81	2.55	7.67	2.50	1.31
Breast	12.83	37.0	48.3	6.77	4.86	4.42	4.72	3.80	6.30	2.49	7.33	2.31	1.26
Loin	18.58	15.8	64.7	6.98	4.90	4.68	5.02	3.98	6.87	2.79	8.00	2.45	1.29
Average ..				7.06	4.97	4.57	4.77	3.82	6.74	2.56	7.65	2.43	1.30
					Cooked samples								
Rib chop	17.20	41.0	41.2	7.62	5.07	4.90	5.05	4.07	6.97	2.76	7.52	2.20	1.32
Loin "	19.06	35.7	44.0	7.76	4.75	4.82	4.92	3.98	7.20	2.87	7.68	2.15	1.25
Shoulder	22.54	18.4	58.5	7.71	4.92	5.06	5.04	3.88	7.23	2.76	7.44	2.25	1.35
Leg	19.84	39.2	40.0	7.63	4.90	4.94	4.78	4.20	7.18	2.67	7.87	2.36	1.27
Breast	20.22	42.3	37.4	8.17	5.29	5.20	5.00	4.24	6.98	2.81	7.72	2.13	1.44
Loin roast	23.85	25.9	48.9	7.77	5.20	4.96	5.08	3.96	6.34	2.94	7.68	2.18	1.35
Average..........				7.78	5.02	4.98	4.98	4.06	6.98	2.80	7.65	2.21	1.33

* The values for protein, ether extract, and moisture are expressed as the percentage in the moist meat and the values for the amino acids are expressed as the percentage in the crude protein (N × 6.25).

of histidine in different cuts of pork varies considerably; namely, 2.72 to 3.56 per cent of the protein for blade steak and loin chop, respectively.

The values for pork and lamb are in good agreement with those obtained previously and by other workers (5, 6, 8, 9, 15–22). Lyman and Kuiken (21) have obtained somewhat higher values for leucine and lysine. They analyzed the lean tissue after removing the separable fat and connective tissue, while the present analyses were made on the entire edible portion of the cuts.

The data confirm previous findings that the amino acids so far studied are essentially stable to cooking. This is evidenced from the fact that no appreciable changes were noted in the percentage of the amino acids in the protein of the fresh and cooked samples (Tables IV and V). The retention of the amino acids in the cooked cuts was also determined by comparing the total amount of each amino acid in the uncooked cuts with the total amount in the cuts after cooking. The percentage retentions obtained showed that no appreciable loss of the amino acids occurred during cooking. For example, the percentage of valine retained averaged 101 per cent (range 97 to 106 per cent), the lysine retained averaged 99 per cent (range 89 to 108 per cent), and the tryptophan retained averaged 104 per cent (range 96 to 115 per cent). These studies are now being extended to include other amino acids and studies on the availability of amino acids in uncooked and cooked meats.

SUMMARY

The amount of leucine, valine, isoleucine, threonine, phenylalanine, arginine, histidine, lysine, methionine, and tryptophan in several cuts of fresh and cooked pork and lamb samples was determined.

Other tests showed that excellent agreement in the assay values for histidine was obtained when either *S. faecalis* R or *L. mesenteroides* P-60 was used as the test organism and when phenylalanine was assayed with either *L. arabinosus* or *L. mesenteroides*. Data obtained by the chemical method for methionine and by microbiological techniques showed that the methods gave similar results for samples with a wide range in methionine content.

The amino acids studied were found to be stable to cooking.

BIBLIOGRAPHY

1. Snell, E. E., *Advances in Protein Chem.*, **2**, 85 (1945).
2. Snell, E. E., *J. Bact.*, **50**, 373 (1945).
3. Schweigert, B. S., and Snell, E. E., *Nutr. Abstr. and Rev.*, **16**, 497 (1946).
4. Schweigert, B. S., *J. Am. Dietet. Assn.*, **24**, 939 (1948).
5. Lyman, C. M., Kuiken, K. A., and Hale, F., *J. Biol. Chem.*, **171**, 233 (1947).
6. Greenhut, I. T., Sirny, R. J., and Elvehjem, C. A., *J. Nutr.*, **35**, 689 (1948).
7. National Cooperative Meat Investigators, Meat and meat cookery, Chicago (1942).
8. Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Strong, F. M., *J. Biol. Chem.*, **155**, 183 (1944).
9. Greenhut, I. T., Schweigert, B. S., and Elvehjem, C. A., *J. Biol. Chem.*, **162**, 69 (1946).
10. Riesen, W. H., Schweigert, B. S., and Elvehjem, C. A., *J. Biol. Chem.*, **165**, 347 (1946).
11. Graham, C. E., Smith, E. P., Hier, S. W., and Klein, D., *J. Biol. Chem.*, **168**, 711 (1947).

12. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).
13. Lyman, C. M., Moseley, O., Wood, S., and Hale, F., *Arch. Biochem.*, **10**, 427 (1946).
14. Csonka, F. A., and Denton, C. A., *J. Biol. Chem.*, **163**, 329 (1946).
15. Schweigert, B. S., Tatman, I. E., and Elvehjem, C. A., *Arch. Biochem.*, **6**, 177 (1945).
16. Hier, S. W., Graham, C. E., Freides, R., and Klein, D., *J. Biol. Chem.*, **161**, 705 (1945).
17. Greenhut, I. T., Schweigert, B. S., and Elvehjem, C. A., *J. Biol. Chem.*, **165**, 325 (1946).
18. Lyman, C. M., Butler, B., Moseley, O., Wood, S., and Hale, F., *J. Biol. Chem.*, **166**, 173 (1946).
19. Greenhut, I. T., Potter, R. L., and Elvehjem, C. A., *Arch. Biochem.*, **15**, 459 (1947).
20. Kuiken, K. A., Lyman, C. M., and Hale, F., *J. Biol. Chem.*, **171**, 561 (1947).
21. Lyman, C. M., and Kuiken, K. A., *Texas Agr. Exp. Sta. Bull. 708* (1949).
22. Kraybill, H. R., in Sahyun, M., Proteins and amino acids in nutrition, New York (1948).

FATTY ACID SYNTHESIS BY ENZYME PREPARATIONS OF CLOSTRIDIUM KLUYVERI*

I. PREPARATION OF CELL-FREE EXTRACTS THAT CATALYZE THE CONVERSION OF ETHANOL AND ACETATE TO BUTYRATE AND CAPROATE

By E. R. STADTMAN AND H. A. BARKER

(From the Division of Plant Nutrition, University of California, Berkeley)

Present knowledge concerning the synthesis of fatty acids is derived mainly from studies with various animals and microorganisms. These studies, facilitated greatly by the use of stable and radioactive isotopes, have established the important fact that fatty acid synthesis in both animals and microorganisms involves a multiple condensation of 2-carbon compounds (2, 6, 7, 8, 11). Almost nothing is known, however, about the mechanism by which this condensation is accomplished. A further insight into the mechanism of this conversion has been clouded by the fact that, in general, the results obtained from studies *in vivo* are complicated by the simultaneous synthesis or decomposition of other metabolites, *viz.* carbohydrates. Moreover, a more detailed study of this problem has not been possible because of the inability to obtain suitable enzyme preparations with which the individual reactions could be studied under controlled conditions.

Several years ago, an anaerobic bacterium, *Clostridium kluyveri*, was discovered, whose energy metabolism was concerned solely with the synthesis of the lower fatty acids (1). Recently this bacterium has been studied extensively, and it has been shown to be able to obtain energy for growth from the conversion of 2-carbon compounds (ethanol and acetate) to fatty acids of 4 and 6 carbon atoms (3, 4). Tracer experiments (2) have shown that this conversion is an oxidation-reduction process in which ethanol is oxidized to a 2-carbon compound ("active" acetate) that is in approximate equilibrium with acetate. This "active" acetate is condensed with acetate to a C_4 compound that serves as the oxidant for the reaction and is reduced to butyrate.
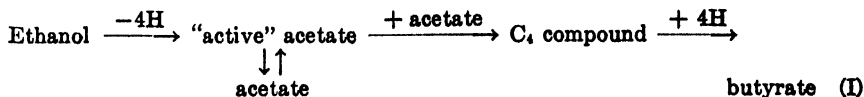
$$\text{Ethanol} \xrightarrow{-4\text{H}} \text{``active'' acetate} \xrightarrow{+ \text{ acetate}} C_4 \text{ compound} \xrightarrow{+ 4\text{H}}$$
$$\updownarrow$$
$$\text{acetate} \qquad\qquad\qquad\qquad\qquad\qquad \text{butyrate} \quad \text{(I)}$$

In a similar manner the "active" acetate derived from another molecule of ethanol may condense with butyrate to form caproate. Thus, in this organism, as in animals (6) and yeast (7, 8, 11), the biological synthesis of fatty acids is accomplished by the multiple condensation of 2-carbon molecules.

C. kluyveri can be grown easily in a completely synthetic medium (3) containing ethanol and a lower fatty acid as the only carbon sources. It cannot utilize other common fermentation substrates such as glucose, pyruvate, or the dicarboxylic acids; therefore, studies on fatty acid synthesis are not complicated by side reactions involving these compounds.

The over-all reactions involved in the synthesis of fatty acids by this organism are well established by chemical data (4) and particularly by data obtained in tracer studies (2, 10). It appeared, therefore, that C. kluyveri would be an excellent biological material to use in the study of the more intimate reactions involved in the synthesis of fatty acids.

The present investigation has shown that cell-free enzyme preparations of C. kluyveri can be obtained that catalyze all the energy-yielding reactions characteristic of the living organism.

Under anaerobic conditions, these enzyme preparations catalyze the complete conversion of ethanol and acetate to butyrate and caproate. Under aerobic conditions, with oxygen serving as the electron acceptor, ethanol is oxidized quantitatively to acetyl phosphate; also under these conditions, fatty acids of 4, 6, or 8 carbon atoms are oxidized to acetyl phosphate and acetate. A condensation of acetyl phosphate and acetate is catalyzed and the C_4 condensation product is reduced to butyric acid. For this reduction and for the reduction of some other 4-carbon compounds studied, molecular hydrogen can serve as the hydrogen donor. With these enzyme preparations the mechanism of fatty acid synthesis has been studied in some detail.

It is the purpose of this paper to describe a method for obtaining active cell-free enzyme preparations of C. kluyveri. In subsequent papers, it will be shown how these preparations have been used to study the individual reactions involved in fatty acid synthesis.

Methods

Enzyme Activity—The oxidation of ethanol and butyrate was determined manometrically by measuring the oxygen consumption in a Warburg apparatus.

Fatty acid synthesis was studied by incubating ethanol and acetate with the enzyme preparation in an evacuated Thunberg tube. After incubation, the presence of butyrate and caproate was detected by Duclaux distillation of the steam-volatile acids. The individual fatty acids were

separated from the mixture by partition chromatographic analysis, by the method of Elsden (5).

Growth of C. kluyveri—Even under optimal conditions, the growth of *C. kluyveri* is relatively slight and the yield of dry cells is only about 150 mg. per liter of culture medium. It was, therefore, desirable to grow the organism on a large scale.

Media found previously to support good growth in 1 liter cultures (3) did not give good growth in 12 to 20 liter cultures. In large cultures, the bacteria formed slimy masses which autolyzed and settled to the bottom of the culture flask. Experiments by B. E. Volcani (unpublished) indicated that this condition was due to an unfavorably high phosphate concentration. By decreasing the phosphate concentration of the medium and by increasing the bicarbonate level to compensate for the loss in buffering capacity, good growth of *C. kluyveri* was obtained in 12 to 200 liter cultures.

The composition of the medium as modified for large scale growth (20 to 200 liter cultures) is as follows: ethanol 20 gm., potassium acetate 4.3 gm., K_2HPO_4-KH_2PO_4 buffer (1 M, pH 7.0) 3.5 ml., acetic acid (glacial) 2.5 ml., $(NH_4)_2SO_4$ 500 mg., $MgSO_4 \cdot 7H_2O$ 200 mg., $CaSO_4 \cdot 2H_2O$ 10 mg., $FeSO_4 \cdot 7H_2O$ 5 mg., $MnSO_4 \cdot 4H_2O$ 2.5 mg., $Na_2MoO_4 \cdot 2H_2O$ 2.5 mg., $Na_2S \cdot 9H_2O$ 200 mg., K_2CO_3 5.9 gm., biotin 10 γ, *p*-aminobenzoic acid 1.0 mg., and tap water (35°) 1000 ml.

All reagents except the sodium sulfide and potassium carbonate were dissolved and the resulting solution was deaerated by passing a vigorous stream of nitrogen through it for several minutes. After deaeration the sodium sulfide and carbonate were added. The pH of the medium was usually about 7.0; otherwise, it was adjusted to this value by adding potassium carbonate or by bubbling in carbon dioxide.

Under anaerobic conditions the above medium is highly selective, and it is possible to grow the organism quite successfully in unsterilized media without significant contamination by other organisms.

Ordinarily, five 20 liter cultures were grown simultaneously and were harvested together. In later experiments, the bacteria were grown in a 55 gallon (208 liter) iron drum, equipped with a 5 cm. opening in the top. 10 volumes per cent of an actively growing culture of *C. kluyveri* served as inoculum.

Growth was determined by measuring the turbidity of 10 ml. aliquots in an Evelyn colorimeter (3).

Since about 10 per cent of the ethanol is converted to acetic acid and hydrogen (4), a large volume of hydrogen is evolved in large scale fermentations. Moreover, appreciable quantities of CO_2 are liberated from the bicarbonate buffer as a result of acid formation. Therefore, gas

evolution, as measured by a "precision" wet test gas meter, was also used to follow the course of the fermentation. When the rate of gas production began to fall off, the bacteria were harvested. As a safety precaution, the evolved gases were directed outside the building.

Gas production and changes in turbidity for a typical 200 liter culture are shown in Fig. 1. In a normal fermentation on this scale, 250 to 350 liters of gas are evolved before the culture is ready to harvest; then the optical density $(2 - \log G$ value$)^1$ of the culture is about 0.130 to 0.175.

Preparation of Dried Cells—When the culture was ready to harvest, the bacteria were separated by centrifugation. The culture solution was siphoned into a Sharples supercentrifuge operating at about 30,000 R.P.M.
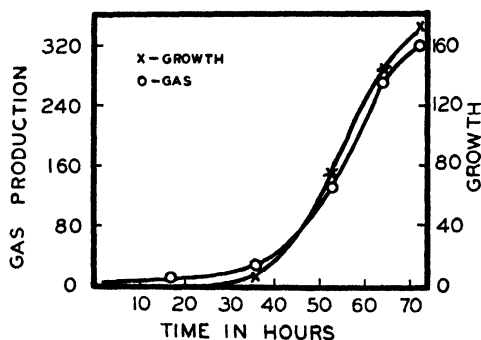
FIG. 1. Hydrogen production and growth of *C. kluyveri* in a 208 liter culture. X refers to growth measured by $(2 - \log G) \times 1000$, where G is the galvanometer reading on an Evelyn colorimeter. O refers to the liters of gas produced. 30–34°.

The rate of flow was about 1 liter per minute. After centrifuging, the compact mass of bacteria from 100 to 200 liters of medium was suspended in 300 ml. of water containing 0.03 per cent $Na_2S \cdot 9H_2O$ (pH 8.0). The pH of this suspension was normally about 6.4 to 6.5 and was immediately adjusted to 7.0 with 1 N NaOH. In some preparations 0.05 M veronal buffer (pH 7.9) was used instead of water.

The thick suspension was transferred to 75 ml. centrifuge tubes and the bacteria were separated by centrifuging in a Servall conical type centrifuge (about 4000 R.P.M.) for 5 minutes. The bacteria were washed two more times in 300 ml. of the dilute sulfide solution. Finally the cells were spread in a thin film (about 3 mm.) in one or more 15 cm. Petri dishes. These were placed in a desiccator over $CaCl_2$ and dried *in vacuo* (2 to 5 mm. of Hg) at room temperature. During evacuation, the cell paste foamed up, forming a large spongy mass that dried in 5 to 8 hours.

Usually about 150 mg. of dry cells were obtained from 1 liter of culture

---

[1] G is the galvanometer reading on an Evelyn colorimeter.

solution. Dried cells not used immediately for the preparation of cell-free extracts were sealed in test-tubes under a vacuum (2 to 5 mm. of Hg) and stored at −18°. The latter precaution is probably not necessary, as the dry cells have been kept for several days at room temperature without appreciable loss in activity of the enzymes studied.

A description of the culturing conditions for several different lots of dried cells used in the present investigation is presented in Table I.

Preparation of Cell-Free Extracts—Cell-free enzyme preparations were obtained by extracting the dry cells with dilute sulfide solution; the insoluble material was separated by centrifuging, and the clear, cell-free

TABLE I

Dried Cell Preparations Used in This Investigation

Lot	Size of growing culture	Age of culture when harvested	Dry weight of bacteria	Dry bacteria per liter
	liters	*hrs.*	*gm.*	*gm.*
C	5 × 20	68	13.1	0.131
D	4 × 20	122	12.6	0.158
E	208	120	44.2	0.220
G	208	120	31.1	0.149
H	5 × 20	74	18.2	0.182
I	208	75	69.8	0.335

supernatant solution was lyophilized. Details of the procedure are as follows:

10 gm. of dried cells were suspended in 150 ml. of freshly deaerated distilled water, containing 0.03 per cent sodium sulfide ($Na_2S \cdot 9H_2O$). The suspension was adjusted to pH 7.0 with 1 N NaOH and allowed to stand at room temperature (20–24°) for 1 to 6 hours with occasional shaking. The suspension was held in a 500 ml. round bottom flask, which was evacuated to keep it anaerobic during the incubation period. After incubation, the suspension was centrifuged 5 to 10 minutes in a refrigerated centrifuge (0°) operating at about 17,000 R.P.M. The clear, cell-free supernatant solution was transferred to a 2 liter round bottom flask that was immediately evacuated. The enzyme solution was frozen by rotating the flask in a dry ice-chloroform bath and was lyophilized at 10^{-2} to 10^{-3} mm. of mercury pressure. With the equipment used, about 8 hours were required to dry 150 ml. of enzyme solution.

The cell débris removed by one centrifugation was resuspended in 150 ml. of sulfide solution. The suspension was again centrifuged and the cell-free extract dried as before. Sometimes a third extraction was also made.

The dried extracts were transferred to test-tubes that were sealed under a vacuum and stored at $-18°$. Cell-free extracts thus stored showed no appreciable loss in activity of the enzymes studied over a period of 6 months.

Effect of Various Extracting Conditions on Enzyme Activity—In preparing the first cell-free extracts by the above procedure, a 1 hour extracting time was used, and highly active enzyme preparations (Lots C and D) were obtained. In later experiments, with the same technique, cell-free extracts were obtained that were relatively inactive (Lots E and E-2). Since the dried cells were very active in both instances, it was apparent that some condition in the extracting procedure was inadequate. Therefore a study was made to determine the effect of various extracting conditions on the activity of the cell-free extracts. For these studies dried cells from Lots E and G were used, as considerable difficulty had been encountered in obtaining active cell-free extracts from these lots.

Effect of pH—Suspensions of dried cells (Lot G) were adjusted to various pH levels (pH 5.2 to 9.6) with 0.1 N HCl or NaOH. After 20 minutes incubation at room temperature the samples were centrifuged, and the cell-free extracts were adjusted to pH 7.9 and tested for their ability to catalyze the oxidation of ethanol and butyrate. The results, summarized in Fig. 2, show that maximal activity was obtained by extracting at pH 6.8.

It is not evident from the experiment whether the lower activities obtained by extracting at more acid or more alkaline pH levels are caused by enzyme inactivation or by poorer extraction. The lower activity at pH 8.0 is probably due mainly to poorer extraction, since it has been shown in other experiments (Paper II of this series (9)) that the optimal pH range for ethanol oxidation by cell-free extracts is 8.1 to 8.5.

Effect of Extracting Time—The influence of extraction time on the activity of the cell-free extracts was studied as follows: 750 mg. of dried cells (Lot G) were suspended in 15 ml. of 0.02 per cent $Na_2S \cdot 9H_2O$. The suspension was incubated in an evacuated Thunberg tube at room temperature. After various time intervals, 2.5 ml. aliquots of the suspension were centrifuged and the cell-free extracts were tested for their ability to catalyze the oxidation of ethanol and butyric acid. The results, summarized in Fig. 3, show that the activity increased rapidly with increasing extraction time up to 2 to 3 hours, when maximal activity was obtained. Further incubation for 8 hours caused no appreciable loss in activity.

These results show quite clearly that the failure to obtain active cell-free extracts in Lots E and E-2 was due to an insufficient incubation period (1 hour) prior to centrifugation. Microscopic examination of the suspension of Lot E after 1 hour's incubation showed that the bacterial cells were still intact. Apparently, a longer incubation period is required to provide

sufficient autolysis to liberate the enzymes from the cells. This conclusion was verified by the observation that only 19 per cent of the dry weight was extracted after 1 hour's incubation, while 53.7 per cent was extracted during a 4 hour period.

In other experiments it was found that the addition of inorganic phosphate (0.006 M) to the extracting solution greatly accelerated the rate of extraction of the enzyme activity but had little influence on the total amount of enzyme extracted. The addition of small amounts of ethanol (0.13 per cent) to the extraction solution also had a slight beneficial effect.
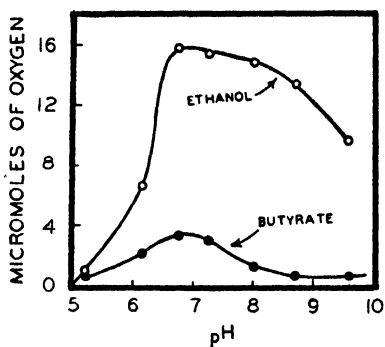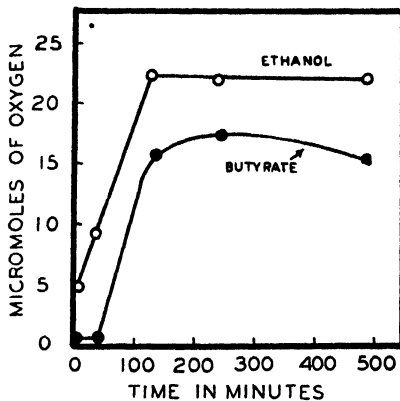
FIG. 2. The influence of the pH of extraction on the activity of cell-free extracts. The ordinate refers to micromoles of oxygen consumed in 20 minutes at 26°. Each Warburg vessel contained 0.1 ml. of 1 M phosphate (pH 7.9), 0.1 ml. of 0.02 M methylene blue, 0.1 ml. of 20 per cent ethanol or 0.5 M sodium butyrate, 0.8 ml. of cell-free extract, and 0.9 ml. of distilled water. The final pH was 7.8 to 8.0 in all vessels.

FIG. 3. The effect of extraction time on the activity of cell-free extracts. Each Warburg vessel contained 0.1 ml. of 0.2 M sodium butyrate or 0.1 ml. of 20 per cent ethanol, 0.1 ml. of 0.02 M methylene blue, 0.1 ml. of 1 M phosphate (pH 8.1), 0.8 ml. of cell-free extract (Lot G), and 0.9 ml. of water. The ordinate refers to micromoles of oxygen consumed at 26°. The abscissa refers to the time of extraction.

It is not understood why an extraction period of 1 hour was sufficient for the preparation of active cell-free extracts of Lots C and D but not of Lots E and G. So far as possible, the method of extracting was identical in all cases. The results emphasize the fact that each lot of dried cells must be tested in order to ascertain how much extraction time is required to obtain maximal activity.

When optimal conditions are used, well over 50 per cent of the enzyme activity of the dried cells is recovered in the dried cell-free extract.

Information concerning the various cell-free extracts used in the present

investigation is presented in Table II. The alphabetic character used to denote a given cell-free extract is the same as that used for the dried cell preparation from which the extract was obtained (see Table I).

TABLE II

Cell-Free Extracts Used in This Investigation

Lots C', D', E', E-2', and G' were obtained by reextracting the cellular débris separated from the first extract. Lot C" + D" was obtained by reextraction of the combined cellular débris from extracts of Lots C' and D'. Lot E-2 was obtained by extracting dried cells of Lot E with a 0.03 per cent ethanol solution.

Lot	Dry cells extracted	Extraction time	Dry weight of extract	Dry extract / Dry cells
	gm.	*hrs.*	*gm.*	
C	13.1	1	4.9	0.370
C'		1		
D	12.6	1	4.0	0.316
D'		1	1.58	
C" + D"		1	1.28	
E	20.0	1	3.3	0.165
E'		1	2.5	
E-2	18.5	1	3.5	0.190
E-2'		1	1.5	
G	20.0	4	10.7	0.535
G'		1	3.2	

TABLE III

Conversion of Ethanol and Carboxyl-Labeled Acetate to Butyrate and Caproate

In a Thunberg tube were placed 0.05 ml. of 20 per cent sodium acetate, 0.1 ml. of 20 per cent ethanol, 0.05 ml. of carboxyl-labeled acetate (12.6 μM = 19,000 counts per minute), 0.5 ml. of 0.02 per cent $Na_2S \cdot 9H_2O$, and 30 mg. of dried cell-free extract. The tube was evacuated and stored at 34° for 13 hours.

		C^{14}
	μM	*counts per min. per μM*
Acetate added	83	229
Volatile acids after incubation.	87	
Acetate recovered	19.3	32
Butyrate "	13.4	221
Caproate "	10.0	382

Fatty Acid Synthesis—When ethanol and acetate were incubated together with the cell-free extracts under anaerobic conditions, it was found that longer chain fatty acids were formed. To prove conclusively that the enzyme preparations actually catalyzed the conversion of ethanol and acetate to these longer chain fatty acids, an experiment was done in which acetic acid labeled in the carboxyl group with C^{14} was incubated with un-labeled ethanol. After incubation, the volatile acids were separated by

partition chromatography. Pure caproic, butyric, and acetic acids were identified by Duclaux distillation. The results of C^{14} measurements are recorded in Table III. All the fatty acids were found to contain large amounts of the isotope. The specific activities of the caproic and butyric acids were 1.7 and 1.0 times as great as the specific activity of the acetate added. The activity of the residual acetate was only about 15 per cent as great as that of the acetate added, showing that the ethanol had been oxidized to acetate or to a substance in isotopic equilibrium with acetate. These results are therefore qualitatively identical with those obtained with growing cultures (2), and they show definitely that cell-free extracts of *C. kluyveri* can catalyze the conversion of ethanol and acetate to butyrate and caproate.

Not all enzyme preparations were equally capable of synthesizing butyrate and caproate from ethanol and acetate. With most preparations that caused little or no synthesis from these substrates, butyric and caproic acids were formed when a little acetyl phosphate was added also. It will be shown in a later paper that acetyl phosphate is the end-product of ethanol oxidation. This oxidation is coupled with the reduction of a C_4 compound produced by the condensation of acetyl phosphate and acetate. Thus the necessity of adding acetyl phosphate to some enzyme preparations is probably due to the fact that these preparations do not contain sufficient amounts of a suitable hydrogen acceptor to initiate the oxidation of ethanol to acetyl phosphate.

SUMMARY

A method has been described for the preparation of cell-free extracts of the bacterium *Clostridium kluyveri* that can catalyze the anaerobic conversion of ethanol and acetate to fatty acids of 4 and 6 carbon atoms. These preparations also catalyze the aerobic oxidation of ethanol and butyrate.

BIBLIOGRAPHY

1. Barker, H. A., *Arch. Mikrobiol.*, **8**, 314 (1937).
2. Barker, H. A., Kamen, M. D., and Bornstein, B. T., *Proc. Nat. Acad. Sc.*, **81**, 373 (1945).
3. Bornstein, B. T., and Barker, H. A., *J. Bact.*, **55**, 223 (1948).
4. Bornstein, B. T., and Barker, H. A., *J. Biol. Chem.*, **172**, 659 (1948).
5. Elsden, S. R., *Biochem. J.*, **40**, 252 (1946).
6. Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **160**, 417 (1945).
7. Smedley-MacLean, I. S., and Hoffert, D., *Biochem. J.*, **17**, 720 (1923).
8. Sonderhoff, R., and Thomas, H., *Ann. Chem.*, **530**, 1195 (1937).
9. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **180**, 1095 (1949).
10. Stadtman, E. R., Stadtman, T. E., and Barker, H. A., *J. Biol. Chem.*, **178**, 677 (1949).
11. White, A. G. C., and Werkman, C. H., *Arch. Biochem.*, **13**, 27 (1937).

FATTY ACID SYNTHESIS BY ENZYME PREPARATIONS OF CLOSTRIDIUM KLUYVERI*

II. THE AEROBIC OXIDATION OF ETHANOL AND BUTYRATE WITH THE FORMATION OF ACETYL PHOSPHATE

By E. R. STADTMAN and H. A. BARKER

(*From the Division of Plant Nutrition, University of California, Berkeley*)

The anaerobic bacterium *Clostridium kluyveri* can catalyze the conversion of ethanol and acetate to butyrate and caproate (6). Tracer studies with growing cultures of this organism (2) have shown that this conversion involves an oxidation of ethanol to a 2-carbon compound ("active" acetate) that is in approximate equilibrium with acetate. This activated 2-carbon substance then condenses with ordinary acetate to form a C_4 compound that is reduced to butyrate (see Scheme I, Paper I of this series). If the current idea is correct that biological oxidation-reduction reactions involve the removal or addition of only 2 electrons at a time, it follows that the conversion of alcohol and acetate to butyrate must involve a number of intermediates. For example, the oxidation of ethanol to the "active" acetate would be a two-step process involving at least one intermediate compound. Similarly, the reduction of the postulated C_4 compound to butyrate should involve at least one intermediate compound in a state of oxidation corresponding to β-hydroxybutyrate. Thus it is necessary to postulate a series of reactions in which at least four intermediate compounds are formed in the conversion of alcohol to butyrate.

$$\text{Ethanol} \xrightarrow{-2H} \begin{array}{c}\text{Compound } a \\ (\text{acetaldehyde ?})\end{array} \xrightarrow{-2H} \begin{array}{c}\text{Compound } b \\ (\text{acetyl P ?})\end{array}$$

$$\downarrow + \text{Acetate} \qquad \text{(I)}$$

$$\begin{array}{c}\text{Butyrate}\end{array} \xleftarrow{+2H} \begin{array}{c}\text{Compound } d \\ (\text{crotonate ?}) \\ (\beta\text{-hydroxybutyrate ?}) \\ (\text{vinyl acetate ?})\end{array} \xleftarrow{+2H} \begin{array}{c}\text{Compound } c \\ (\text{acetoacetate ?})\end{array}$$
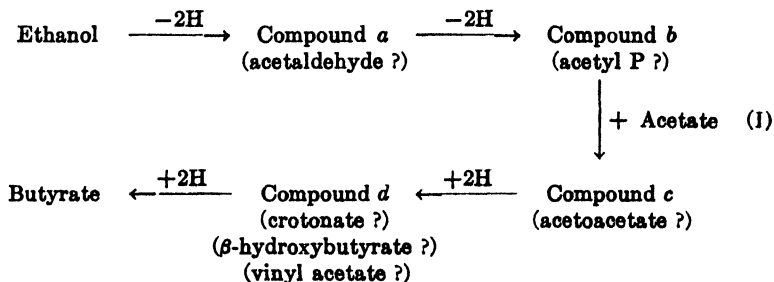
Bornstein and Barker (6) postulated that Compound a is acetaldehyde. Acetaldehyde is an intermediate in the oxidation of ethanol to acetate by

acetic acid bacteria (37), and the enzymes catalyzing the oxidation of ethanol to acetaldehyde are found in animal tissues (8).   Barker (1) postulated that Compound b is acetyl phosphate, since *Clostridium butylicum*, an organism that also produces butyric acid from acetate (46), had been shown to produce acetyl phosphate or a similar compound (20). The intermediate, Compound c, should be a C_4 compound in the same state of oxidation as acetoacetate.   Acetoacetate is a common end-product of fatty acid oxidation by higher animals and is formed by the condensation of 2-carbon molecules (45).   To date, however, there is no direct evidence that acetoacetate is an actual intermediate in fatty acid synthesis.   Crotonic, β-hydroxybutyric, and vinylacetic acids are 4-carbon compounds in a state of oxidation corresponding to Compound d.   All three of these substances have been studied extensively (27, 16, 23, 19), but the rôle that they may play in fatty acid synthesis is still uncertain.   Examination of Scheme I shows that, while Compound b is the final end-product of ethanol oxidation, it cannot possibly accumulate *in vivo*, since it must condense with acetate to form Compounds c and d which are the main oxidants in the system.   If an electron acceptor other than Compound c could be found, it should be possible to obtain an oxidation of ethanol with an accumulation of Compounds a and b.   So far all such attempts with growing cultures of *C. kluyveri* have been unsuccessful (5).   Since the organism is a strict anaerobe, the use of oxygen as an electron acceptor *in vivo* is excluded.

In Paper I of this series (41), it was shown that cell-free enzyme preparations of *C. kluyveri* will, under anaerobic conditions, catalyze the conversion of ethanol and acetate to butyrate and caproate.   These enzyme preparations also catalyze the aerobic oxidation of ethanol and butyrate when oxygen is used as the electron acceptor.

With oxygen serving as the electron acceptor, the oxidation of ethanol should lead to the accumulation of the intermediates Compounds a, b, and possibly Compound c.   If the reactions shown in Scheme I are reversible, butyrate oxidation should also lead to an accumulation of these intermediates plus acetate.

It is the purpose of the present paper to describe the results of studies on the aerobic oxidation of ethanol and butyrate by enzyme preparations of *C. kluyveri*.   These studies have confirmed the hypothesis that ethanol is oxidized to acetyl phosphate and that acetaldehyde is an intermediate in this oxidation (1).   Also, as predicted, butyrate is oxidized almost quantitatively to a mixture of acetyl phosphate and acetate.

Methods

Total volatile acids were estimated by steam distillation and titration. Separation of the fatty acids in mixtures was accomplished by partition

chromatographic analysis (11), and the pure acids were identified by Duclaux distillation. In some experiments in which the fatty acid mixture was composed of acetic, butyric, and caproic acids only, the concentrations were calculated from Duclaux distillation data.

Acetaldehyde was estimated by the bisulfite-binding method of Elliott *et al.* (10) and also by microtitration of the acid liberated when acetaldehyde was distilled from the test solution (pH 7.0) into hydroxylamine hydrochloride (33).

Three methods were used to determine inorganic phosphate. (*a*) When no acyl phosphate was present, the inorganic phosphate was estimated directly on a deproteinized trichloroacetic acid filtrate by means of the Fiske-Subbarow procedure (12). (*b*) When acyl phosphates were present, the calcium precipitation method of Lipmann and Tuttle (28) was used. (*c*) The inorganic phosphate content of synthetic acetyl phosphate preparations was determined by the method of Lowry and Lopez (31).

Acyl phosphates were estimated by the specific colorimetric method of Lipmann and Tuttle (29). In some instances, methylene blue was present in the samples to be tested. This was removed by extracting with butanol after the hydroxamic acid had been converted to the colored iron complex. Three successive extractions with an equal volume of butanol were sufficient to remove all of the methylene blue. The hydroxamic acid complex was not extracted by this procedure. The resulting solution was made up to the original volume with distilled water.

The disilver salt of monoacetylphosphoric acid was prepared by the method of Bentley (3). The silver salt was subsequently converted to the lithium salt by the addition of lithium chloride and the lithium salt was purified by precipitation from alcoholic solution as described by Lipmann (30). The lithium salt (about 95 per cent pure) was used directly.

The highly specific acetylphosphatase was prepared from horse muscle by the method of Lipmann (unpublished; *cf.* (26)).

The oxidation of ethanol, acetaldehyde, and fatty acids was estimated by measuring the oxygen uptake manometrically in a Warburg apparatus. Air was the gas phase and the temperature was 26°.

Veronal buffer (0.04 M, pH 8.1) was found not to inhibit the enzymes involved in ethanol or acetaldehyde oxidation and was generally used when the oxidation of these substrates was studied. However, veronal did inhibit the oxidation of fatty acids and therefore tris(hydroxymethyl)aminomethane (13) (0.05 M, pH 7.6 to 8.1) was used when the oxidation of fatty acids was investigated.

A description of the various enzyme preparations used in these investigations has been given in Paper I (41).

Results

Factors Influencing Oxidation of Ethanol—In preliminary experiments a rapid oxidation was observed only during the first few minutes of incubation. The rate fell off rapidly and the oxidation sometimes ceased before appreciable quantities of ethanol had been oxidized. This inactivation was correlated with changes in pH and with exposure of the enzyme to oxygen in the absence of a substrate. Therefore, the influence of these factors on the oxidation of ethanol was studied in more detail.
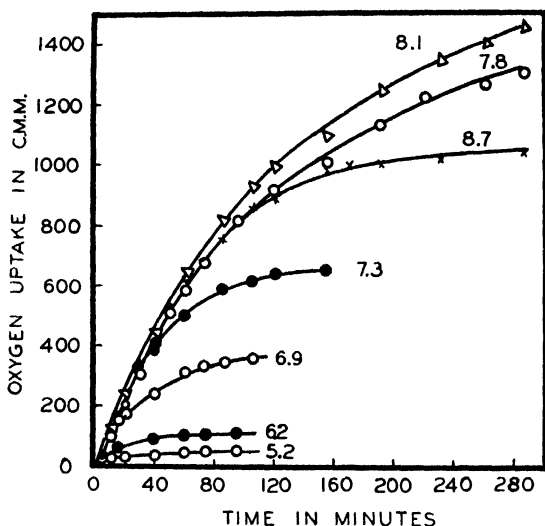
FIG. 1. The influence of pH on the oxidation of ethanol. Each Warburg vessel contained 0.05 M phosphate buffer and 0.04 M veronal buffer at the various pH levels, 0.001 M methylene blue, 25 mg. of enzyme (Lot C), and 500 μM of ethanol as substrate. The enzyme was dissolved in 1.0 ml. of water and dumped from the side arm after equilibration at 26°. The gas phase was air. The numbers on the various curves indicate the initial pH of the reaction mixture.

Effect of pH—The influence of pH is shown in Fig. 1. Although the initial rate of oxidation was essentially the same over the pH range of 6.9 to 8.7, marked differences were observed in the rate of inactivation. A rapid inactivation occurred at pH levels below 7.3. Maximal stability was present in the range of pH 7.8 to 8.1. At pH 8.7 inactivation occurred only after prolonged incubation.

Effect of Oxygen—Complete inhibition of the ethanol-oxidizing system occurs if the enzyme preparations are shaken in air for 5 minutes in the absence of an oxidizable substrate. No such inhibition occurs even after prolonged shaking (1 hour) in nitrogen, or after shaking in air when ethanol

is present. Therefore, in measuring the oxidation of ethanol, the enzyme solution was always placed in the side arm of the Warburg vessel and dumped into the substrate after equilibration rather than vice versa. By confining the enzyme solution in the narrow side arm of the vessel, relatively little surface is exposed to the air during the equilibration and almost no inactivation occurs. Whenever possible, the enzyme was dissolved in the largest convenient volume of water (usually 1.0 ml.) so that the ratio of liquid volume to exposed surface would be at a maximum. In some experiments the enzyme was mixed directly with the substrate and the oxygen uptake during the equilibration period was determined by extrapolation.
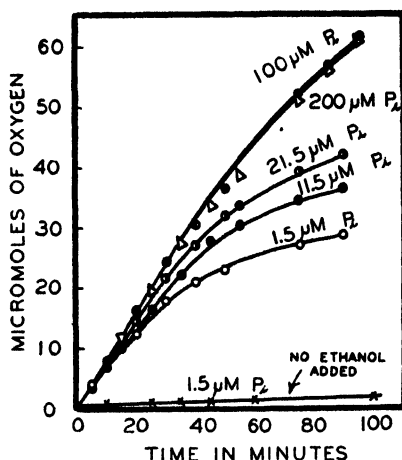
Fig. 2. The effect of inorganic phosphate on the oxidation of ethanol. Each Warburg vessel contained 0.04 M veronal buffer (pH 8.1), 500 μM of ethanol, 50 mg. of dried cells (Lot I), and the indicated quantities of inorganic phosphate (Pi). The total liquid volume was 2.0 ml. The dried cells were suspended in 1.0 ml. of water and tipped into the substrate after 10 minutes equilibration at 26°. The ordinate refers to micromoles of oxygen consumed.

Influence of Inorganic Phosphate on Oxidation of Ethanol—The results of a typical experiment, presented in Fig. 2, show clearly that the amount of oxidation (oxygen uptake) is a function of the phosphate concentration. There was a progressive increase in the total quantity of oxygen consumed as the phosphate level was increased from 1.5 to 100 μM per 2 ml. Increasing the phosphate level to 200 μM (0.1 M) caused no further increase in rate of oxidation or in the total amount of oxygen consumed. Of particular interest is the observation that 29 μM of oxygen were consumed even when no inorganic phosphate was added. The inorganic phosphate content of the enzyme preparation (1.5 μM) was insufficient to account for the observed

oxygen consumption if ethanol oxidation was always coupled with an esterification of inorganic phosphate.

The results suggested that the oxidation of ethanol does not require inorganic phosphate but that the primary product of oxidation is not further oxidized unless inorganic phosphate is provided. If the mechanism postulated in Scheme I is correct, the oxidation of ethanol in the absence of inorganic phosphate should result in an accumulation of acetaldehyde and no acetyl phosphate or acetic acid should be formed.

TABLE I

Oxidation of Ethanol to Acetaldehyde in Absence of Inorganic Phosphate

Each Warburg vessel contained 0.04 M veronal buffer (pH 8.1) and 50 mg. of dried cells (Lot I) in a total liquid volume of 2.0 ml. The enzyme was added to the substrate just before placing the vessels in the water bath at 26°. The oxygen utilization for the first 10 minutes was therefore determined by extrapolation to zero time. The total incubation time was 205 minutes.

)strate	$-O_2$	Acetaldehyde*	Δ volatile acids†	$-O_2$ calculated‡
	microatoms	*micromoles*	*μM*	*microatoms*
Ethanol, 500 μM. . . .	79.4	54	9.1	72.2
Acetaldehyde, 104 μM . .	4.9	98		
None (control).	4.6	0		

* Estimated by the hydroxylamine hydrochloride method.

† Determined in a parallel experiment; expressed as increase in volatile acid above the control.

‡ The calculation is based on the assumption that 2 microatoms of oxygen are used in the formation of 1 μM of volatile acid, and that 1 microatom is used for each micromole of acetaldehyde produced.

Oxidation of Ethanol to Acetaldehyde—In order to test the above possibility, ethanol was oxidized in the absence of inorganic phosphate and the reaction mixture was then examined for acetaldehyde and volatile acids. A second sample containing acetaldehyde as the only substrate was used as a control. The results are shown in Table I. As predicted, there was a large accumulation of acetaldehyde (54 μM), accounting for at least 82 per cent of the ethanol oxidized. Only 9 μM of volatile acid were produced, showing that very little further oxidation occurred when no inorganic phosphate was added. The results demonstrate clearly that in the absence of inorganic phosphate ethanol is oxidized more or less quantitatively to acetaldehyde according to reaction (1).

$$C_2H_5OH + \tfrac{1}{2}O_2 \rightarrow CH_3CHO + H_2O \qquad (1)$$

Oxidation of Ethanol to Acetyl Phosphate—The discovery that inorganic phosphate is not necessary for the oxidation of ethanol to acetaldehyde

but, nevertheless, does increase the total oxygen consumption, indicated that inorganic phosphate is concerned with the further oxidation of acetaldehyde, probably to acetyl phosphate. The oxidation of ethanol in the presence of inorganic phosphate therefore was studied more extensively in order to determine the products of oxidation under these conditions. In a number of experiments the inorganic phosphate concentration, the period of incubation, and the pH of the enzyme suspension were varied. Since it was anticipated that acetate or acetyl phosphate would be formed, the oxidation products were analyzed for total volatile acids, acyl phosphate, and inorganic phosphate. Typical results are summarized in Table II.

TABLE II

Oxidation of Ethanol in Presence of Inorganic Phosphate

Each Warburg vessel contained 500 μM of ethanol, 0.001 M methylene blue, 0.04 M veronal buffer (at the pH indicated), 23 mg. of dried cell-free enzyme preparation (Lot C), and the indicated amounts of inorganic phosphate. The total liquid volume was 2.0 ml. The enzyme preparation dissolved in 1.0 ml. was dumped from the side arm after equilibration. The temperature was 26°. After incubating in air for the period of time indicated, the mixture was analyzed for inorganic phosphate, acyl phosphate, and volatile acid.

pH	Incubation time	P_i, initial*	Δ Pi†	Δ Pac‡	$-O_2$	Δ volatile acids§	$\dfrac{\Delta \text{ Pac}}{-O_2}$	$\dfrac{\Delta \text{ Pac}}{\Delta \text{ Pi}}$	$\dfrac{\Delta \text{ volatile acid}}{-O_2}$
	min.	μM	μM	μM	μM	μM			
8.1	105	11.4		10.1	20.1	7.2	0.50		0.35
8.1	260	52.4	41.3	40.8	58.0	45.4	0.74	0.99	0.78
6.8	105	103	21.0	12.5	16.2	13.4	0.77	0.60	0.82
7.8	385	103	48.4	48.0	62.5	65.2	0.78	0.99	1.04
8.1	385	103	55.8	53.4	66.6	68.6	0.80	0.95	1.03
8.7	385	103	37.6	40.1	47.5	54.4	0.84	1.06	1.13

* Includes the inorganic phosphate present in the enzyme preparation (1.4 μM).
† Determined by the method of Lipmann and Tuttle (28).
‡ Determined by the method of Lipmann and Tuttle (29).
§ This value includes acetyl phosphate and acetate.

These data show that when adequate amounts of inorganic phosphate are added 1 mole of acetyl phosphate is produced and 1 mole of inorganic phosphate is esterified for each mole of oxygen utilized. In experiments to be described later, it was shown that the acyl phosphate produced was monoacetyl phosphate. The reaction may therefore be represented by the equation

$$C_2H_5OH + O_2 + HPO_4^- \rightarrow CH_3COOPO_3^- + H_2O \qquad (2)$$

Examination of the last column in Table II reveals that the ratio of volatile acids produced (*i.e.* acetyl phosphate + acetate) to the oxygen con-

sumed increased as the initial inorganic phosphate level was increased. A ratio of 1.0 was obtained at the highest levels. This indicates that at high phosphate levels little or no acetaldehyde accumulates and the over-all reaction is quantitatively described by reaction (2). At the lower phosphate levels the ratio was appreciably less than unity. Considerable amounts of acetaldehyde must have accumulated in these samples, since the molar quantity of oxygen consumed exceeded the quantity of inorganic phosphate added. The acetyl phosphate produced represents 75 to 85 per cent of the total volatile acid. In a subsequent paper, it is shown that the
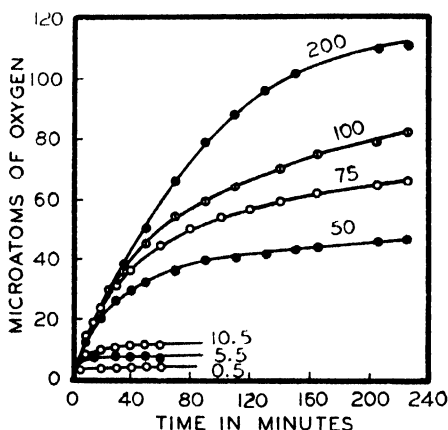
FIG. 3. The influence of inorganic phosphate concentration on the oxidation of acetaldehyde. Each Warburg vessel contained 150 μM of acetaldehyde, 0.04 M veronal buffer (pH 8.1), 0.001 M methylene blue, and 20 mg. of cell-free extract (Lot D). The total liquid volume was 2.3 ml. The enzyme preparation was dissolved in 1.0 ml. of water and dumped from the side arm after equilibration at 26°. The gas phase was air. The numbers on the various curves indicate the micromoles of inorganic phosphate added.

small amount of free acetic acid undoubtedly is formed by hydrolysis of acetyl phosphate by an acylphosphatase present in the cell-free extract.

Oxidation of Acetaldehyde to Acetyl Phosphate—From the above studies it appeared that the formation of acetyl phosphate from ethanol (reaction (2)) is in reality a two-step process in which acetaldehyde is the intermediate compound whose further oxidation is coupled with an esterification of inorganic phosphate. In order to determine whether this was the case, the dependence of acetaldehyde oxidation on the concentration of inorganic phosphate was studied. The results given in Fig. 3 show that acetaldehyde is oxidized only in the presence of inorganic phosphate. At the lower phosphate levels (5.5 to 50 μM), at which the uptake of oxygen had almost ceased by the end of the incubation period, there was good agreement between

the molar quantity of oxygen consumed and the amount of inorganic phosphate added.

After incubation, the contents of the Warburg vessels were analyzed for acetyl phosphate, inorganic phosphate, and total volatile acid. The data in Table III show that for each atom of oxygen consumed 1 mole of acetyl phosphate was produced, and they provide final evidence that equations (1) and (3) delineate the mechanism of ethanol oxidation.

$$CH_3CHO + \tfrac{1}{2}O_2 + HPO_4^= \rightarrow CH_3COOPO_3^= \tag{3}$$

Effect of Ratio of Inorganic Phosphate to Acetyl Phosphate on Rate of Acetaldehyde Oxidation—Examination of Fig. 3 shows that the rate of acetaldehyde oxidation decreases gradually with increase in incubation time.

TABLE III

Oxidation of Acetaldehyde to Acetyl Phosphate

For the experimental conditions see Fig. 3.

Pi initial	Δ Pi*	Δ Pact	O₂ uptake	Volatile acids‡	$\dfrac{\Delta Pac}{O_2 \text{ uptake}}$	$\dfrac{\Delta Pac}{\Delta Pi}$
μM	μM	μM	microatoms	μM		
0.5	0.25		3.5			
5.5	4.4	6.1	6.9	5.7	0.88	1.48
10.5	8.9	10.7	10.7	9.0	1.00	1.21
50.5	41.5	46.6	46.8	48.1	0.97	1.12
75.5	68.3	68.8	66.2	69.1	0.99	1.01
100	75.7	85.1	82.2	81.4	1.04	1.12
200	84.5	93.1	111	97.6	0.96	1.12

* Determined by the method of Lipmann and Tuttle (28).
† Determined by the method of Lipmann and Tuttle (29).
‡ The value includes acetyl phosphate and acetate.

Moreover, the rate of decrease is greater at the lower phosphate levels. Since the initial rate of oxidation is essentially identical over the range of phosphate levels varying from 50 to 200 μM, it is obvious that this decline in rate is not due to a decreasing phosphate concentration. As the initial acetaldehyde concentration was the same in all samples, the change in rate was clearly not due to a change in substrate concentration. If the effect were due to enzyme inactivation, one would expect inactivation to occur in all samples at the same rate. Since this did not occur, it seemed likely that the change in rate was due to inhibition by the product of the oxidation, acetyl phosphate. To test this possibility the rates of oxidation at various times, estimated from the corresponding tangents to the curves in Fig. 3, were plotted as a function of the ratio of inorganic phosphate to acetyl phosphate (Pi:Pac). The inorganic phosphate-acetyl phosphate ratio at

any given period of time was calculated from oxygen uptake data. The results of three sets of calculations based on the oxygen uptake-time curves for the samples initially containing 50, 75, and 100 μM of inorganic phosphate are plotted in Fig. 4. Since all three sets of data fall on the same curve, it is obvious that the decrease in rate of acetaldehyde oxidation was due to the accumulation of acetyl phosphate.

This conclusion was subsequently tested experimentally by measuring the rate of acetaldehyde oxidation in samples to which various amounts of syn-
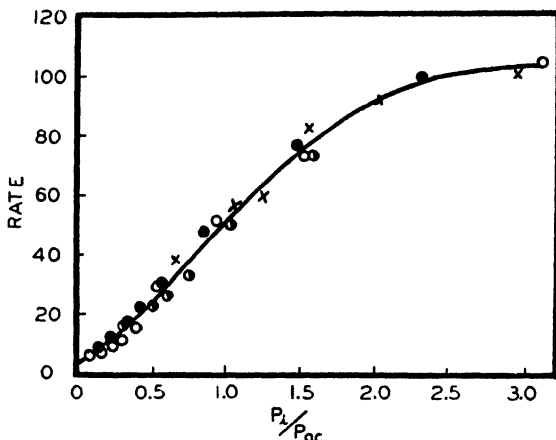
FIG. 4. The influence of the ratio of inorganic phosphate to acetyl phosphate on the rate of acetaldehyde oxidation. The circles refer to values calculated from the data presented in Fig. 3; O, to the sample containing 50 μM of inorganic phosphate (Pi) initially; ●, to the sample containing 75 μM of Pi; ◑ to the sample containing 100 μM of Pi. The rate, expressed in c.mm. of O_2 per 10 minutes, was calculated from tangents to the curves in Fig. 3 (see the text). X represents experimental values obtained from an experiment in which the rate of acetaldehyde oxidation was studied at various acetyl phosphate levels. The latter experiment was similar to that described in Fig. 3, except that each Warburg vessel contained 50 μM of Pi, 100 μM of acetaldehyde, and the amounts of acetyl phosphate indicated by the Pi:Pac ratio. The c.mm. of O_2 consumed during the first 10 minutes of incubation was taken as a measure of the rate.

thetic acetyl phosphate had been added. Sufficient amounts of acetyl phosphate were added so that the Pi:Pac ratio varied from 0.6 to 3.0. The quantity of oxygen consumed in the first 10 minutes of incubation (after equilibration) was taken as a measure of the rate.

The data obtained from this experiment (also plotted in Fig. 4) were in complete agreement with those obtained from Fig. 3. Fig. 4 shows that the rate of oxidation is almost directly proportional to the Pi:Pac ratio over the range from 0 to 2.0, while at higher ratios the rate is essentially constant.

Dismutation of Acetaldehyde—Higher plants (34), animals (8), and bacteria (38) are able to catalyze the dismutation of acetaldehyde to ethanol and acetic acid. Since no steam-volatile acid other than acetic acid is produced in the aerobic oxidation of acetaldehyde by enzyme preparations of *C. kluyveri*, it seemed probable that if a dismutation of acetaldehyde occurred it would involve an esterification of inorganic phosphate according to the reaction,

$$2CH_3CHO + HPO_4^- \rightarrow CH_3COOPO_3^- + C_2H_5OH \tag{4}$$

To investigate this possibility two samples of an enzyme solution, one containing acetaldehyde and one containing ethanol, were incubated in the presence of inorganic phosphate in evacuated Thunberg tubes. After vari-
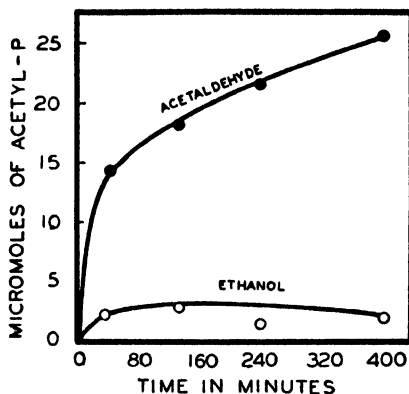
Fig. 5. The dismutation of acetaldehyde. Two Thunberg tubes containing 0.1 M phosphate buffer (pH 7.0), 30 mg. of cell-free extract (Lot C), and 400 μM of ethanol or 150 μM of acetaldehyde in a total liquid volume of 2.0 ml. were evacuated and incubated at 26°.

ous periods of time aliquots were withdrawn and analyzed for acetyl phosphate. The data, presented in Fig. 5, show that acetyl phosphate was formed rapidly from acetaldehyde under anaerobic conditions. The relatively small amount of acetyl phosphate found with ethanol as the substrate was due undoubtedly to the presence of traces of oxidizable substances in the enzyme preparation (see Paper III of this series). The results strongly indicate that a dismutation has occurred according to equation (4), although the formation of alcohol has not been directly demonstrated.

Substitution of Arsenate for Inorganic Phosphate in Oxidation of Acetaldehyde—In several biological reactions involving inorganic phosphate, arsenate can replace phosphate (44, 9, 18). This replacement was investigated in the oxidation of acetaldehyde. The results of a typical experiment, presented in Fig. 6, show that arsenate (0.00025 to 0.025 M) can substitute

for inorganic phosphate to a limited extent. The initial rates of oxidation with arsenate and with phosphate were identical; however, arsenate caused a complete inhibition of oxidation after 15 to 20 minutes incubation.

In the presence of arsenate acetaldehyde was converted quantitatively to acetic acid; no acetyl derivative could be detected. The behavior of arsenate in this system is therefore analogous to its behavior in the oxidation of pyruvic acid by enzyme preparations of *Lactobacillus delbrueckii* (24) and in the oxidation of triose phosphate by yeast preparations (44).
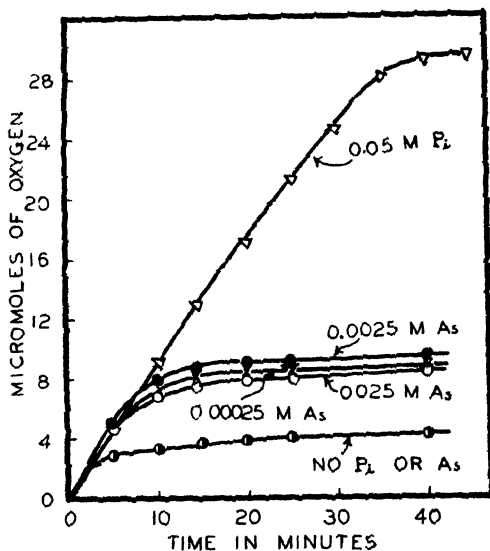
Fig. 6. The effect of arsenate on the oxidation of acetaldehyde. Each Warburg vessel contained 0.04 M veronal buffer (pH 8.7), 55 μM of acetaldehyde, 50 mg. of dried cells (Lot I), and the indicated amounts of arsenate or inorganic phosphate (Pi). The dried cells were suspended in 1.0 ml. of water and dumped from the side arm after equilibration at 26°.

Oxidation of Butyrate to Acetyl Phosphate and Acetate—It has been shown previously (41) that enzyme preparations of *C. kluyveri* are able to convert ethanol and acetate to butyrate and caproate under anaerobic conditions. It seemed possible, therefore, that the preparations would catalyze the oxidation of butyrate and caproate if an oxidant of sufficiently high potential was used. Inasmuch as molecular oxygen can serve as an oxidant for ethanol and acetaldehyde, the enzyme preparations contain an active terminal oxidase system. It appeared probable, therefore, that butyrate could be oxidized aerobically. This was confirmed by observing the uptake of oxygen manometrically when butyrate was added to the enzyme preparation.

It was found (Fig. 7) that inorganic phosphate is required for butyrate oxidation. No significant oxygen uptake occurred unless inorganic phosphate was added. Arsenate was completely ineffective in replacing inorganic phosphate.

If the mechanism of butyrate oxidation were the reverse of that postulated in Scheme I for the synthesis of butyrate, one would expect to find acetyl phosphate and acetate as the final end-products. Therefore, several experiments were performed to determine whether acetyl phosphate and acetate are actually formed. The results of a typical experiment, given in
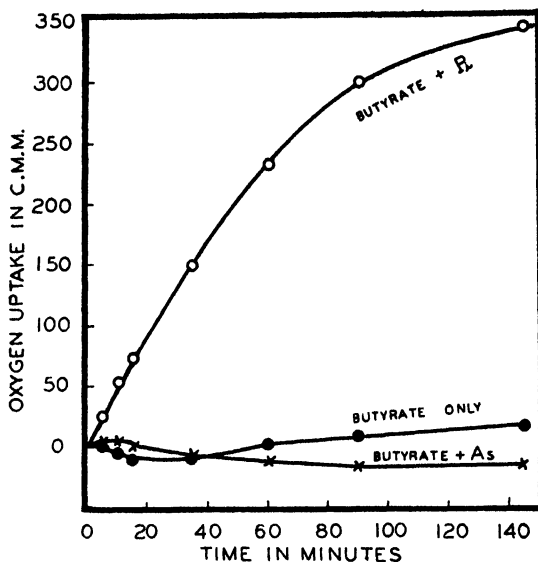
FIG. 7. The effect of inorganic phosphate and arsenate on the oxidation of butyrate. Each Warburg vessel contained 50 mg. of dried cells (Lot H), 40 μM of butyrate, and the indicated amount of inorganic phosphate or arsenate. The total liquid volume was 2.0 ml. The pH was 8.0. The samples were incubated at 26° in air.

Table IV, show that for each mole of butyrate added 1 mole of oxygen was consumed and 1 mole of acetyl phosphate and volatile acid was formed. The volatile acid was shown by Duclaux distillation to be acetic acid. These results may be represented by equation (5).

$$CH_3CH_2CH_2COO^- + O_2 + HPO_4^- \rightarrow CH_3COOPO_3 + CH_3COO^- + H_2O \quad (5)$$

The butyrate-oxidizing system was rather insensitive to the hydrogen ion concentration over the range from pH 7.0 to 8.0, but the rate of oxidation fell off sharply on either side of this range; almost no oxidation was observed at pH 5.0 or 9.2.

The system was completely insensitive to cyanide at concentrations from 0.02 to 0.08 M.

Oxidation of Fatty Acids Other Than Butyrate—Growing cultures of *C. kluyveri* are able to synthesize fatty acids with 4 to 7 carbon atoms (6). It

TABLE IV

Oxidation of Butyrate to Acetyl Phosphate and Acetate

Each Warburg vessel contained 50 mg. of enzyme, 100 μM of inorganic phosphate (pH 8.1); 0.1 ml. of NaOH (3.5 M) was placed in the center wells. The total liquid volume was 2.0 ml. All samples were incubated 45 minutes at 26°, after which time the oxygen uptake had stopped. All data have been corrected for a relatively small blank (about 10 per cent) obtained when similar samples were incubated in the absence of added butyrate.

Enzyme preparation	Na butyrate added	$-O_2$	Δ acetyl P	Δ volatile acids*	$\dfrac{\Delta \text{ acetyl P}}{-O_2}$
	μM	μM	μM	μM	
Lot I, dried cells	18.2	18.5	17.7	17.7	0.96
" G, cell-free extract	18.2	18.2	16.6	15.5	0.91

* Total volatile acid minus acetyl phosphate. Duclaux distillation of the volatile acid proved it to be pure acetic acid.

TABLE V

Oxidation of Various Fatty Acids (C_4 to C_{10})

Each Warburg vessel contained 0.1 M inorganic phosphate, 25 mg. of dried cells (Lot H), and about 10 μM of fatty acid. The total liquid volume was 2.0 ml.; temperature, 26°. All values for oxygen uptake have been corrected for a blank sample to which no substrate was added.

Fatty acid	O_2 uptake in 25 min
	c.mm
Butyric	224
Valeric	225
Caproic	180
Heptanoic	86
Octanoic	76
Capric	−6

was of interest, therefore, to see whether the enzyme preparation could oxidize fatty acids containing 5 or more carbon atoms.

The results of a single experiment in which the oxidation of fatty acids containing 4 to 10 carbons was tested are reported in Table V. Butyrate and valerate were oxidized at the same rate. Increasing the chain length above 5 carbon atoms caused the rate to fall off sharply. During the 25

minute incubation period only about one-third as much oxygen was consumed with heptanoate or octanoate as with butyrate; capric acid was not oxidized at all.

The oxidation of octanoate is of interest, since this acid apparently is not formed in any significant amount by growing cultures of *C. kluyveri* (6). Heptanoate, which is formed by growing cultures from ethanol and propionate, is not oxidized by the enzyme preparation at an appreciably greater rate than is octanoate.

The relative rates of oxidation of the fatty acids reported in Table V refer to the rates obtained when all fatty acids were present initially at a concentration of 0.005 M. The lower rates observed with the longer chain fatty acids may be due to an inhibitory effect of these acids when present in a relatively high concentration. Such an inhibitory effect was observed by Grafflin and Green (15) in studies with rabbit kidney preparations. In other experiments it was shown that enzyme preparations of *C. kluyveri* did not oxidize capric acid in 0.0005 M concentrations. The failure to obtain oxidation apparently was not due to an inhibition of the enzyme system, since the rate of butyrate oxidation was unaffected by the addition of capric acid (0.0005 to 0.0025 M).

The bacterial enzyme seems, therefore, to be specific for those fatty acids of 8 or fewer carbon atoms. Nonylic acid was not tested. A high degree of specificity has been observed also with enzyme complexes derived from various animal tissues. Thus the rabbit kidney complex (15) was able to oxidize fatty acids containing 12 or fewer carbon atoms (C_{10} and C_{12} only at very low concentrations), whereas the rat liver complex studied by Lehninger (21) was able to oxidize all the fatty acids from C_4 to C_{18}, and the rate of oxidation actually increased with an increase in chain length.

Effect of Dialysis on Oxidation of Butyrate—Washed particulate enzyme suspensions derived from rat liver (22) will oxidize fatty acids only if adenylic acid (or a suitable derivative), inorganic phosphate, and fumarate (or another 4-carbon dicarboxylic acid) are added. It has been shown that the oxidation of butyrate by enzyme preparations of *C. kluyveri* requires the addition of inorganic phosphate; however, attempts to stimulate oxidation by the addition of adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, acetyl phosphate, or fumarate were unsuccessful. It was possible that sufficient quantities of such substances were present in the enzyme preparation. To investigate this possibility, studies were made with dialyzed extracts. The dialysis was carried out in two steps, the first of which consisted of a crude dialysis against a relatively small volume of dilute sulfide solution. A concentrated dialysate was thus obtained, which could be used to reactivate the dialyzed enzyme. The second step was a more exhaustive dialysis against a large volume of sulfide solution. In

both instances the dialysis was made under strictly anaerobic conditions. More complete details are as follows:

Preparation of Dialysate—1.0 gm. of cell-free extract (Lot G) dissolved in 10 ml. of 0.02 per cent $Na_2S \cdot 9H_2O$ was sealed in a cellophane bag. The bag was placed in a 35 ml. glass vial which was completely filled with dilute sulfide solution. The vial was sealed with a rubber stopper and was shaken vigorously on a shaking machine, overnight, at 4°. The dialysate (about 25 ml.) was a brilliant yellow with a strong greenish fluorescence, typical of the flavins. It was further concentrated *in vacuo* to a volume of 10 ml.

Preparation of Dialyzed Enzyme—After the preliminary dialysis described above, the enzyme solution (still in the cellophane bag) was transferred to a 1 liter Erlenmeyer flask completely filled with the dilute sulfide solution. The flask was stoppered and the dialysis was continued for 6 hours at 4° with vigorous stirring by means of an induction type stirrer. The dialyzed enzyme thus obtained was still faintly yellow, indicating that not all of the flavin had been removed. Also, it had become somewhat cloudy in appearance, indicating some protein denaturation.

The dialyzed enzyme preparation was almost completely unable to catalyze the oxidation of butyrate but was partially activated by the addition of inorganic phosphate. Attempts were made to stimulate the oxidation further by the addition of the dialysate, yeast extract, and acetyl phosphate. The results, given in Table VI, show that yeast extract alone had no activating effect. However, when acetyl phosphate was added in addition to yeast extract, oxidation was increased 3-fold. The dialysate was slightly less effective than yeast extract and apparently supplied the same or similar factors needed for butyrate oxidation, since little further improvement was obtained when yeast extract and the dialysate were added simultaneously.

Since no significant amount of acetyl phosphate could be detected in the undialyzed preparations, the observation that acetyl phosphate is needed to stimulate butyrate oxidation by dialyzed extracts was unexpected. The obvious conclusion was that undialyzed preparations contain acetyl phosphate in trace amounts that cannot be determined by the usual methods of analysis. However, all attempts to inactivate the butyrate oxidase system in undialyzed preparations by preincubating them with a highly active animal acetylphosphatase were unsuccessful.

A number of experiments were done to see whether some of the common coenzymes could substitute for the dialysate or yeast extract. The data from a typical experiment, given in Table VII,[1] show that none of the sub-

[1] The data of Table VII are not directly comparable with those of Table VI, since the dialyzed enzyme preparation had been kept in the refrigerator 2 days, during which time some inactivation had taken place. Also the concentration of the enzyme used in the last experiment was only two-thirds as great as in the experiment described in Table VI.

stances tested (coenzyme I, adenosine triphosphate, adenosine monophosphate, flavin-adenine dinucleotide, or any of the divalent ions) could replace the dialysate. It is also evident that both acetyl phosphate and the dialysate must be present in order to obtain activation; little or no stimulation was obtained when either one was added separately.

In other experiments that will not be described in detail, the effects of the various coenzymes added independently and in various combinations were studied. In no instance was it possible to show a stimulation with anything other than acetyl phosphate and the dialysate or yeast extract.

TABLE VI

*Effect of Yeast Extract, Acetyl Phosphate, and Dialysate on Oxidation of Butyrate by Dialyzed Extracts of C. kluyveri**

Each Warburg vessel contained 0.5 ml. of the dialyzed enzyme solution (about 50 mg., dry weight), 40 μM of sodium butyrate, and the amounts of inorganic phosphate, yeast extract, dialysate, and acetyl phosphate indicated. The total volume was 2.0 ml.; temperature, 26°.

Inorganic phosphate added	Yeast extract added†	Dialysate added	Acetyl phosphate added	−O₂ in 50 min.
μM	*mg.*	*ml.*	μM	*c.mm.*
0	0	0	0	10
100	0	0	0	114
100	15	0	0	81
100	15	0	10	310
100	0	1.0	10	265
100	15	1.0	10	391

* Unfortunately, in this experiment, no sample was included to show the effect of acetyl phosphate in the absence of yeast extract or dialysate. It is evident from the data of Table VII that acetyl phosphate is ineffective unless yeast extract or dialysate is added simultaneously.

† The yeast extract was prepared as follows: 100 gm. of dry yeast (Fleischmann's, 20-40) suspended in 300 ml. of water were incubated at 37° for 90 minutes and centrifuged (about 2500 R.P.M.) for 30 minutes. The slightly turbid supernatant solution was lyophilized and the supply of dried extract thus obtained was stored at −18°.

In view of the fact that Mg^{++} is reported necessary for the oxidation of fatty acids by animal enzyme systems (22, 15, 35), this ion was given special attention but no activation could be observed.

The dialysate obviously contains some as yet unidentified factor or factors involved in fatty acid oxidation. Until this factor is more adequately characterized, the negative results obtained with the other cofactors studied are not conclusive. Some of these factors may be required in addition to the unknown factors in the dialysate.

Identification of Monoacetyl Phosphate As End-Product of Ethanol, Acetal-

dehyde, and Butyrate Oxidation—The following evidence indicates that monoacetyl phosphate is the end-product of these oxidations. (*a*) The

TABLE VII

Effect of Various Coenzymes on Activity of Dialyzed Extracts

Each Warburg vessel contained 0.5 ml. of dialyzed enzyme (Lot G), 100 μM of inorganic phosphate, 40 μM of butyrate, and the amounts of other substances indicated. The total volume was 3.0 ml.; temperature, 26°.

Acetyl P added	Divalent ions added,* 0.1 per cent solution	Coenzyme I added, 0.1 per cent solution	Flavin-adenine dinucleotide added	Adenosine monophosphate added	Adenosine triphosphate added	Dialysate added	Boiled dialysate added	Yeast extract added	O₂ uptake
μM	*ml.*	*ml.*	γ	μM	μM	*ml.*	*ml.*	*mg.*	*c.mm.*
0	0	0	0	0	0	1	0	0	15
10	0	0	0	0	0	0	.0	0	12
10	0	0	0	0	0	1	0	0	106
10	0	0	0	0	0	0	1	0	124
10	0.1	0.1	0	0	0	0	0	0	17
10	0.1	0.1	20	0	0	0	0	0	8
10	0.1	0.1	20	10	0	0	0	0	7
10	0.1	0.1	20	10	10	0	0	0	3
10	0.1	0.1	20	10	10	1	0	0	56
10	0.1	0.1	20	10	10	1	0	15	169

* A mixture of $MgSO_4$, $MnSO_4$, $FeSO_4$, $ZnSO_4$, $CoCl_2$, and $CaCl_2$.

TABLE VIII

Hydrolysis of Natural and Synthetic Acetyl Phosphate

Time	Acid hydrolysis*		Enzymatic hydrolysis†	
	Natural	Synthetic	Natural	Synthetic
min.	*per cent*	*per cent*	*per cent*	*per cent*
5	23	23	28	27
10	42	40	50	52
15	52	55	62	64
20	65	66		
25	72	74		
30	79	80		85
40	88	87		

* Hydrolysis in 0.5 N HCl at 40°.

† Hydrolysis by purified muscle acetyl phosphatase. Acetate buffer (0.1 M, pH 5.6); 37°; initial inorganic phosphate, 0.012 M.

substance reacts readily with hydroxylamine to give a hydroxamic acid under the conditions of Lipmann and Tuttle (29). (*b*) Steam distillation

of samples, in which these substrates were oxidized more or less quantitatively to an acyl phosphate (Tables II, III, and IV), showed that 1 mole of volatile acid is obtained per mole of acyl phosphate formed; a Duclaux distillation of the volatile acid showed it to be acetic acid. (c) The rate of hydrolysis in 0.5 N hydrochloric acid at 40° is identical with that of synthetic monoacetyl phosphate (Table VIII). (d) The effect of synthetic monoacetyl phosphate on the rate of acetaldehyde oxidation is exactly the same as that of the compound produced enzymatically (see Fig. 5). (e) The rate of hydrolysis by the highly specific acetylphosphatase obtained from horse muscle (26, 17) is identical with that for monoacetyl phosphate (Table VIII).

DISCUSSION

The experiments described above have shown that enzyme preparations of *C. kluyveri* catalyze the oxidation of ethanol to acetyl phosphate. The oxidation proceeds in two steps: (a) the ethanol is oxidized to acetaldehyde, and (b) the acetaldehyde is further oxidized in the presence of inorganic phosphate to acetyl phosphate. No oxidation of acetaldehyde occurs in the absence of inorganic phosphate or arsenate.

The oxidation of ethanol to acetaldehyde is catalyzed by animals (32), plants (42), and microorganisms (36). Several different kinds of enzymes catalyzing the oxidation of acetaldehyde to acetate have been described (14, 8, 39). However, a coupled oxidative phosphorylation of acetaldehyde with the formation of acetyl phosphate has not been reported previously, and, in those instances in which this point was specifically tested, it was found that inorganic phosphate definitely was not required for the oxidation of acetaldehyde (8, 39). The oxidation of acetaldehyde to acetyl phosphate is, therefore, a new type of acetaldehyde oxidation. In most other reactions in which the formation of acetyl phosphate is coupled with the esterification of inorganic phosphate, pyruvate serves as the starting material. Thus enzyme preparations of *Lactobacillus delbrueckii* catalyze the oxidative decarboxylation of pyruvate to acetyl phosphate and CO_2 (24, 25); extracts of *Escherichia coli* catalyze the phosphoroclastic splitting of pyruvate to acetyl phosphate and formate (43); and dried extracts of *Clostridium butylicum* catalyze the oxidation of pyruvate to acetyl phosphate, H_2, and CO_2 (20). The only other substrate known to be decomposed with the formation of acetyl phosphate is acetoacetate (40). The phosphoroclastic splitting of acetoacetate will be discussed more extensively in a later paper.

The oxidation of acetyldehyde to acetyl phosphate is analogous to the oxidation of triose phosphate to 1,3-diphosphoglyceric acid (44). In the latter reaction it is postulated that 1,3-diphosphoglyceraldehyde is the actual reductant. Lipmann (24) has postulated an analogous intermediate

in the oxidative formation of acetyl phosphate from pyruvate. Similarly, one may postulate that 1-phosphoacetaldehyde is an intermediate in acetaldehyde oxidation (equation (6)).

$$CH_3CHO + HPO_3^- \longrightarrow CH_3-\underset{\underset{H}{|}}{\overset{\overset{OPO_3^-}{|}}{C}}-OH \xrightarrow{+\frac{1}{2}O_2} CH_3COOPO_3^- + H_2O \quad (6)$$
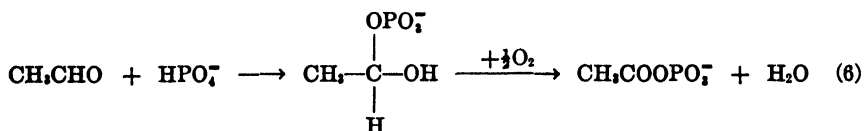
The discovery that ethanol is oxidized to acetyl phosphate is of considerable interest, since it is a confirmation of the idea that an "active" 2-carbon compound is involved in fatty acid synthesis. This idea is further emphasized by the finding that enzyme preparations of *C. kluyveri* catalyze the aerobic oxidation of butyrate to acetyl phosphate and acetate. The latter discovery is a direct confirmation of the fact, already evident from tracer studies with homogenates of animal tissue (7, 45), that fatty acids containing an even number of carbon atoms are oxidized to 2-carbon compounds.

The finding that acetyl phosphate is essential for the oxidation of butyrate by dialyzed enzyme preparations was completely unexpected. It calls to mind the situation encountered with animal fatty acid oxidase systems in which a 4-carbon dicarboxylic acid, such as fumarate, must be added along with adenylic acid, or a suitable derivative, to initiate fatty acid oxidation (15, 22). It is tempting to speculate that the rôle of fumarate and adenylic acid in the animal enzyme systems is, at least in part, analogous to that of acetyl phosphate in the bacterial enzyme system. Both may serve as a source of high energy phosphate or acetyl groups that may be needed in catalytic amounts to initiate the oxidation of fatty acids.

SUMMARY

Cell-free enzyme preparations of *C. kluyveri* have been shown to catalyze the oxidation of ethanol to acetaldehyde. The acetaldehyde thus formed is further oxidized in the presence of inorganic phosphate to monoacetyl phosphate.

The enzyme preparations also catalyze the oxidation of the lower fatty acids. Butyrate is oxidized quantitatively to a mixture of monoacetyl phosphate and acetate.

The oxidation of butyrate by dialyzed enzyme preparations is stimulated by the addition of yeast extract and acetyl phosphate. Attempts to substitute known coenzymes and divalent ions for these materials were unsuccessful.

BIBLIOGRAPHY

1. Barker, H. A., *Antonie van Leeuwenhoek*, **12**, 167 (1947).
2. Barker, H. A., Kamen, M. D., and Bornstein, B. T., *Proc. Nat. Acad. Sc.*, **31**, 373 (1945).
3. Bentley, R., *J. Am. Chem. Soc.*, **70**, 2183 (1948).
4. Berger, L., Slein, M. W., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29**, 379 (1946).
5. Bornstein, B. T., and Barker, H. A., *J. Bact.*, **55**, 223 (1948).
6. Bornstein, B. T., and Barker, H. A., *J. Biol. Chem.*, **172**, 659 (1948).
7. Buchanan, J. M., Sakami, W., Gurin, S., and Wilson, D. W., *J. Biol. Chem.*, **159**, 695 (1945).
8. Dixon, M., and Lutwak-Mann, *Biochem. J.*, **31**, 1347 (1937).
9. Doudoroff, M., Barker, H. A., and Hassid, W. Z., *J. Biol. Chem.*, **170**, 147 (1947).
10. Elliott, K. A. C., Benoy, M. P., and Baker, Z., *Biochem. J.*, **29**, 1937 (1935).
11. Elsden, S. R., *Biochem. J.*, **40**, 252 (1946).
12. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
13. Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, **62**, 33 (1946).
14. Gordon, A. H., Green, D. E., and Subrahmanyan, J., *Biochem J.*, **34**, 764 (1940).
15. Grafflin, A. L., and Green, D. E., *J. Biol. Chem.*, **176**, 95 (1948).
16. Jowett, M., and Quastel, J. H., *Biochem J.*, **29**, 2143 (1935).
17. Kaplan, N. O., and Lipmann, F., *Federation Proc.*, **7**, 163 (1948).
18. Katz, J., Hassid, W. Z., and Doudoroff, M., *Nature*, **161**, 96 (1948).
19. Kleinzeller, Z., *Biochem. J.*, **37**, 678 (1943).
20. Koepsell, H. J., Johnson, M. J., and Meek, J. S., *J. Biol. Chem.*, **154**, 535 (1944).
21. Lehninger, A. L., *J. Biol. Chem.*, **161**, 437 (1945).
22. Lehninger, A. L., and Kennedy, E. P., *J. Biol. Chem.*, **173**, 753 (1948).
23. Leloir, L. F., and Muñoz, J. M., *J. Biol. Chem.*, **153**, 53 (1944).
24. Lipmann, F., *Cold Spring Harbor Symposia Quant. Biol.*, **7**, 248 (1939).
25. Lipmann, F., *J. Biol. Chem.*, **155**, 55 (1944).
26. Lipmann, F., *Advances in Enzymol.*, **6**, 231 (1946).
27. Lipmann, F., and Perlmann, G. E., *Arch. Biochem.*, **1**, 41 (1942-43).
28. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **153**, 571 (1944).
29. Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **159**, 21 (1945).
30. Lipmann, F., and Tuttle, L. C., *Arch. Biochem.*, **13**, 373 (1947).
31. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162**, 421 (1946).
32. Lutwak-Mann, C., *Biochem. J.*, **32**, 1364 (1938).
33. Marasco, M., *Ind. and Eng. Chem.*, **18**, 701 (1926).
34. Michlin, D., and Borodina, O. J., *Enzymologia*, **9**, 28 (1940).
35. Muñoz, J. M., and Leloir, L. F., *J. Biol. Chem.*, **147**, 355 (1943).
36. Negelein, E., and Wulff, H. J., *Biochem. J.*, **289**, 436 (1936); **293**, 351 (1937).
37. Neuberg, C., and Nord, F. F., *Biochem. Z.*, **133**, 96 (1919).
38. Neuberg, C., and Windisch, F., *Biochem. Z.*, **166**, 454 (1925).
39. Racker, E., *J. Biol. Chem.*, **177**, 883 (1949).
40. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **174**, 1039 (1948).
41. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **180**, 1085 (1949).
42. Thunberg, T., *Biochem. Z.*, **206**, 109 (1929).
43. Utter, M. F., and Werkman, C. H., *Arch. Biochem.*, **2**, 491 (1943).
44. Warburg, O., and Christian, W., *Biochem. Z.*, **303**, 40 (1939).
45. Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **155**, 143 (1944).
46. Wood, H. G., Brown, R. W., Werkman, C. H., and Stuckwisch, C. G., *J. Am. Chem. Soc.*, **66**, 1812 (1944).

FATTY ACID SYNTHESIS BY ENZYME PREPARATIONS OF CLOSTRIDIUM KLUYVERI*

III. THE ACTIVATION OF MOLECULAR HYDROGEN AND THE CONVERSION OF ACETYL PHOSPHATE AND ACETATE TO BUTYRATE

By E. R. STADTMAN and H. A. BARKER

(From the Division of Plant Nutrition, University of California, Berkeley)

It has been shown that cell-free enzyme preparations of the bacterium *Clostridium kluyveri* catalyze the anaerobic conversion of ethanol and acetate to butyrate and caproate (5). Under aerobic conditions, with oxygen as the oxidant, these preparations catalyze the oxidation of ethanol to acetyl phosphate and of butyrate to a mixture of acetyl phosphate and acetate (6). These results indicate that acetyl phosphate is an intermediate in the synthesis of butyrate from ethanol and acetate.

$$\text{Ethanol} + \text{H}_3\text{PO}_4 \xrightarrow{-4\text{H}} \text{acetyl phosphate} \xrightleftharpoons[\quad]{\substack{+4\text{H} \\ +\text{acetate}}} \text{butyrate} + \text{H}_3\text{PO}_4 \qquad (\text{I})$$
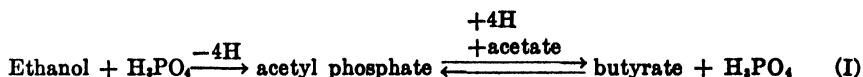
It remains to be demonstrated, however, that acetyl phosphate is actually converted to butyrate. To do this, it would be desirable to use a reducing agent, the presence of which could not complicate the interpretation of the experimental results. Ethanol is the normal hydrogen donor in butyrate synthesis by *C. kluyveri*, but this substance is unsuited for the immediate problem because acetyl phosphate is the end-product of ethanol oxidation. The present investigation was therefore made to find some substance other than ethanol that could act as a hydrogen donor in the enzyme system of *C. kluyveri*. Reduced benzoyl viologen, hydrogen sulfide, and molecular hydrogen were among the substances tried. Indications were obtained that all three of these substances could serve as hydrogen donors. For obvious reasons, molecular hydrogen would be the most suitable and the use of this substance as a hydrogen donor has been studied in some detail.

It is the purpose of this paper to present the results of experiments showing that molecular hydrogen is activated by cell-free extracts of *C. kluyveri* and that, with this substance as a reductant, acetyl phosphate and acetate are converted to butyrate and caproate.

Methods

Hydrogen uptake was measured manometrically by the usual Warburg technique. The other methods used, *i.e.*, the estimation of acyl phosphates and volatile acids and the separation and identification of the lower fatty acids, have been described elsewhere (5, 6). The preparation of the cell-free extracts and a description of the various preparations used in this investigation have also been given elsewhere (5).

Results

Activation of Molecular Hydrogen—Bornstein and Barker (2) have shown that growing cultures of *C. kluyveri* produce small quantities of hydrogen in ethanol-acetate fermentations. This indicates that the bacteria contain an active hydrogenase system catalyzing the reaction,

$$\text{Coenzyme} \cdot 2H \rightleftarrows H_2 + \text{coenzyme} \tag{1}$$
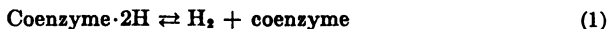
If this is true and if the hydrogenase is not inactivated during the preparation of the cell-free extracts, molecular hydrogen should be able to serve as a reductant in fatty acid synthesis by the enzyme preparations.

Acetaldehyde is an established intermediate in the oxidation of ethanol by these preparations (6) and it should serve as an oxidant under appropriate conditions. Therefore, an experiment was made to determine whether molecular hydrogen could be used to reduce acetaldehyde. The enzyme preparation without added substrate was first shaken in a hydrogen atmosphere until no further uptake of hydrogen occurred (Fig. 1). About 5 μM of hydrogen were consumed during this preliminary incubation period (80 minutes). Then 24 μM of acetaldehyde were added. Hydrogen uptake was immediately resumed at a rapid rate and continued in a linear fashion until 20.4 μM of hydrogen had been used. This corresponds to 85 per cent of the amount required for a reduction of acetaldehyde to ethyl alcohol according to equation (2).

$$CH_3CHO + H_2 \rightarrow CH_3CH_2OH \tag{2}$$

This experiment shows conclusively that molecular hydrogen is activated by the enzyme preparation.

Reduction of Acetyl Phosphate and Acetate with Hydrogen—Since the fact has been established that hydrogen can serve as a reductant in reactions catalyzed by enzyme preparations of *C. kluyveri*, it became possible to test directly the hypothesis that acetyl phosphate and acetate are precursors of butyrate.

When acetyl phosphate and acetate were incubated with the enzyme in a hydrogen atmosphere, hydrogen was consumed at an almost constant rate for 6 hours. Then the experiment was discontinued and the reaction mix-

ture was examined for the presence of butyric acid. A similar sample containing acetaldehyde as the substrate was included for comparison.
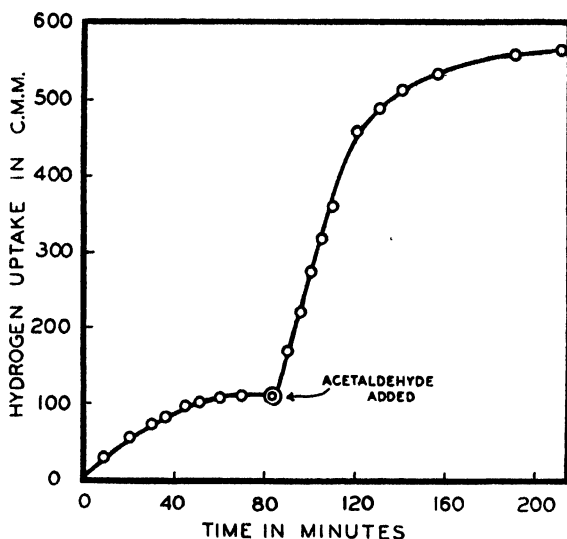
FIG. 1. The reduction of acetaldehyde with molecular hydrogen. The Warburg vessel contained, initially, 0.04 M veronal buffer (pH 8.1) and 23 mg. of cell-free extract (Lot D) in a volume of 2.0 ml. The sample was shaken in hydrogen (26°) until no further uptake of H_2 was observed (70 minutes). At this time, 24 μM of acetaldehyde were introduced into the side arm and the vessel was again flushed with H_2 and after a 5 minute equilibration the acetaldehyde was tipped into the enzyme.

TABLE I

Reduction of Acetaldehyde and Acetyl Phosphate with H_2

Each Warburg vessel contained 0.04 M veronal buffer (pH 8.1) and 20 mg. of cell-free extract (Lot C). The total volume was 2.0 ml. The gas phase was H_2. The total incubation time (at 26°) was 400 minutes. The data have been corrected for the H_2 uptake (3.9 μM), final butyrate content (about 4 μM), and total volatile acid (6 μM) of a control sample incubated without a substrate. The butyrate concentration was computed from Duclaux distillation data.

Substrate	$-H_2$	Total volatile acid, final	Butyrate	$\frac{\text{Butyrate}}{-H_2}$
	μM	μM	μM	
Acetaldehyde, 96 μM.....................	48	3		
Acetyl P, 26 μM, + acetate, 75 μM...........	37	80	17	0.45

The results, presented in Table I, show that for every 2 moles of hydrogen consumed, about 1 mole of butyrate was formed. The data are thus con-

sistent with the view that acetyl phosphate and acetate are reduced to butyrate.

$$CH_3COOPO_3^- + CH_3COO^- + 2H_2 \rightarrow CH_3CH_2CH_2COO^- + HPO_4^- + H_2O \quad (3)$$

Since only 3 μM of volatile acid were produced in the sample containing acetaldehyde, it is probable that most, if not all, of the hydrogen consumed was used to form ethanol (equation (2)).

Influence of Acetate on Synthesis of Butyrate by H_2 Reduction—The foregoing experiment was discontinued before the hydrogen uptake had ceased, and, although 26 μM of acetyl phosphate were added, only 17 μM of butyrate were formed. In other experiments which were allowed to go to completion (to be described, Table II) it was found that acetyl phosphate was reduced almost quantitatively (80 to 95 per cent) according to equation (3). From this it may be concluded that butyrate synthesis involves a net utilization of 1 mole each of acetyl phosphate and acetate. This was to be expected in view of the fact that 1 mole each of these substances was produced in the oxidation of butyrate. The data of these experiments do not, however, indicate how the two C_2 compounds are converted to butyrate. Two obvious mechanisms are possible. 1 molecule of acetyl phosphate may condense with acetate to form an oxidant (Compound c, equation (4)) which is reduced to butyrate. The over-all reaction would be described by equation (3).

$$CH_3COOPO_3^- + CH_3COO^- \longrightarrow \text{Compound } c \xrightarrow{+4H} CH_3CH_2CH_2COO^-$$
$$+ HPO_4^- \quad (4)$$

Another possibility is the condensation of 2 molecules of acetyl phosphate at the expense of only one high energy phosphate. The second phosphate group may be retained to facilitate the subsequent reduction to butyrate and may be used finally to regenerate a mole of acetyl phosphate from ordinary acetate. This mechanism could be described by the following equations.

$$2CH_3COOPO_3^- + 2H_2 \rightarrow CH_3CH_2CH_2COOPO_3^- + HPO_4^- + H_2O \quad (5)$$

$$CH_3CH_2CH_2COOPO_3^- + CH_3COO^- \rightarrow CH_3COOPO_3^- + CH_3CH_2CH_2COO^- \quad (6)$$
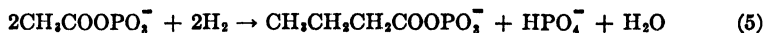
The net result of these two successive reactions is identical with equation (3).

Although butyryl phosphate was used in equations (5) and (6) to represent the hypothetical phosphorylated intermediate, some other substance might serve equally well. The important distinction between the two mechanisms outlined above lies in the fact that in the latter instance 2 molecules of acetyl phosphate condense to form the oxidant, while in the former 1 mole of acetyl phosphate and 1 mole of acetate condense.

It would seem to be a simple matter to differentiate between these two mechanisms. If butyrate is formed only by a condensation of acetyl phosphate and acetate, then no butyrate should be formed when acetyl phosphate is incubated with the enzyme in the absence of acetate. However, it was virtually impossible to study the reduction of acetyl phosphate in the complete absence of acetate, because the enzyme preparations contained small quantities of acetate and because acetyl phosphate is slowly hydrolyzed to acetate, spontaneously and by enzyme action.

If acetyl phosphate and acetate are involved in the primary condensation, the rate of reduction should be influenced by the concentration of acetate.

TABLE II

Effect of Acetate Concentration on Conversion of Acetate and Acetyl Phosphate to Butyrate

Each Warburg vessel contained 0.04 M veronal buffer (pH 8.1), 23 mg. of cell-free extract (Lot D), and the indicated amount of acetyl phosphate and acetate in a total volume of 2.0 ml. The gas phase was H_2. The acetyl phosphate and acetate were tipped into the enzyme after 80 minutes equilibration at 26°, at which time the enzyme preparation was saturated with hydrogen. The total incubation time was 4 hours.

Acetyl P, initial[a]	Acetate, initial[b]	Rate of H_2 uptake, initial	$-H_2$ total	$-H_2$
μM	μM	c.mm. per 10 min.	μM	per cent theory
0	46	2	0.6	
9.6	6	34	16.1	84.0
9.6	26	41	14.9	77.5
9.6	155	53	18.5	96.5
9.6	305	47	18.1	94.2
9.6	455	40	18.1	94.2

[a] Determined in a control sample immediately after mixing with the enzyme preparation.

[b] Includes the acetate present in the enzyme preparation (4 μM) and in the acetyl phosphate preparation (2 μM) after the 80 minute equilibration period.

Therefore, the effect of acetate on the rate of acetyl phosphate reduction was determined. The results (Table II) show clearly that acetyl phosphate must be present to obtain hydrogen uptake; practically no hydrogen was consumed when acetate was the sole substrate. In the presence of acetyl phosphate the rate of hydrogen uptake was influenced only slightly by acetate concentration. Thus, the initial rate of H_2 uptake and the total amount of hydrogen consumed were significantly smaller at the lowest acetate level. The effect was small, however, and on the basis of these data it is not possible to differentiate between the two proposed mechanisms.

In this connection the data obtained in isotope studies with growing cultures of *C. kluyveri* are noteworthy. Barker *et al.* (1) found that, when

ethanol and carboxyl-labeled acetate were fermented, the butyrate formed was labeled in the carboxyl and β positions only. Furthermore, the carboxyl group contained 55 per cent of the activity, whereas the other 45 per cent was located in the β position. Since the activity was not equally distributed between the carboxyl and β positions, the butyrate could not have been produced by a condensation of 2 similar molecules (*viz.* 2 molecules of acetyl phosphate). The observation that more activity was in the carboxyl group is consistent with the theory described by equation (4) if one assumes that complete equilibration between acetyl phosphate and acetate did not take place.

Consideration of Acetaldehyde As Intermediate in Synthesis of Butyrate— It has been suggested that acetaldehyde may condense to acetaldol, which is then converted to butyrate (4). Such a theory is in complete agreement with the over-all quantitative aspects of butyric acid fermentation, and, while some serious objections have been raised against it (3), the possibility that it is correct has not been definitely excluded. The reduction of acetyl phosphate and acetate to butyrate in the foregoing experiments indicates that acetaldehyde is not involved in butyrate synthesis. However, since acetaldehyde can be oxidized to acetyl phosphate (6), it is conceivable that the synthesis of butyrate from acetyl phosphate involves a preliminary reduction of acetyl phosphate to acetaldehyde (equation (7)) which is then converted to butyrate.

$$CH_3COOPO_3^= + H_2 \rightarrow CH_3CHO + HPO_4^= \qquad (7)$$

Against this view are the data of Table I, showing that acetaldehyde is not converted to butyrate under anaerobic conditions. These data are from an experiment in which acetaldehyde was incubated in the absence of inorganic phosphate, and they do not exclude the possibility that a condensation product of acetaldehyde could be converted to butyrate if inorganic phosphate was available. Studies on the conversion of acetaldehyde to butyrate in the presence of inorganic phosphate are complicated by the dismutation of acetaldehyde, with the formation of acetyl phosphate (6).

More convincing evidence against the acetaldehyde hypothesis was obtained by a differential inactivation of the enzymes involved in butyrate and acetaldehyde oxidation (Table III). When the enzyme preparation was shaken in air for several minutes in the absence of an oxidizable substrate, the ability to oxidize acetaldehyde was destroyed, whereas the ability to oxidize butyrate was reduced only slightly. Since there is much evidence that the synthesis of butyrate from acetyl phosphate and acetate occurs by a mechanism that is the exact reverse of the oxidation of butyrate, the fact that butyrate can be oxidized rapidly to acetate and acetyl

phosphate under conditions in which acetaldehyde cannot be oxidized is proof that acetaldehyde is not an obligatory intermediate in butyrate synthesis.

Synthesis of Caproate from Acetyl Phosphate—As noted earlier (5), the enzyme preparations were able to produce significant quantities of caproic acid from ethanol and acetate. Tests were made to determine whether caproic acid could be found in the volatile acid fraction of samples obtained from the hydrogen reduction of acetyl phosphate and acetate. To obtain sufficient material for analysis, the samples from several manometric experiments of the type described in Table II were consolidated. Two lots of samples were used. Lot A contained samples obtained by incubating

TABLE III

Differential Inactivation of Acetaldehyde and Butyrate Oxidation by Aeration of Enzyme Preparation in Absence of Substrate

Aeration before adding substrate	Acetaldehyde			Butyrate		
	$-O_2$	Acetyl P	$\frac{\text{Acetyl P}}{-O_2}$	$-O_2$	Acetyl P	$\frac{\text{Acetyl P}}{-O_2}$
min.	μM	μM		μM	μM	
0	13.4	26.4	1.97	21.6	18.3	0.85
20	2.3	5.0	2.18	19.7	17.8	0.90
40	0.7	2.3	3.3	14.3	11.9	0.83

Each Warburg vessel contained 0.05 M phosphate (pH 7.6), 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 7.6), 50 mg. of dried cells, (Lot I), and 70 μM of acetaldehyde or 40 μM of butyrate as indicated. The total volume was 2.0 ml.; temperature, 26°. The total incubation time after addition of substrate was 40 minutes. All data have been corrected for oxygen uptake (1.2 μM) and acetyl phosphate (1.1 μM) in a control sample without added substrate.

acetyl phosphate with the enzyme preparation; Lot B was from samples to which both acetyl phosphate and acetate had been added. The steam-volatile acids of both lots were fractionated by silica gel partition chromatography, and the various acids were identified by their R_F values and Duclaux distillation. Lot A, to which no acetate had been added initially, contained almost equal amounts of caproic, butyric, and acetic acids. Lot B on the other hand contained only butyric and acetic acids; no caproic acid could be detected. Thus, in the presence of an excess of acetate, the reduction goes almost quantitatively to butyrate. However, when acetate is limiting, caproate is formed also.

Effects of Arsenate and Fluoride—Since arsenate inhibits the oxidation of butyrate (6), tests were made to determine whether it also inhibits butyrate synthesis. It was found that arsenate (0.025 M) caused an almost

complete inhibition of hydrogen uptake when acetyl phosphate and acetate were the substrates. Examination of the enzyme mixture after the experiment revealed that all of the acetyl phosphate had been hydrolyzed. This indicated that the inhibition of arsenate was due, at least in part, to a catalytic action on the decomposition of acetyl phosphate. The effect of arsenate on the dephosphorylation of acetyl phosphate will be discussed further in another paper.

Fluoride (0.02 M) was found not to affect the rate of butyrate synthesis.

SUMMARY

Enzyme preparations of *Clostridium kluyveri* contain an active hydrogenase system. In a hydrogen atmosphere, the preparations catalyze the reduction of acetaldehyde, presumably to ethanol, and the reduction of acetyl phosphate and acetate to butyrate and caproate.

BIBLIOGRAPHY

1. Barker, H. A., Kamen, M. D., and Bornstein, B. T., *Proc. Nat. Acad. Sc.*, **31**, 373 (1945).
2. Bornstein, B. T., and Barker, H. A., *J. Biol. Chem.*, **172**, 659 (1948).
3. Johnson, M. J., Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, **101**, 145 (1933).
4. Kluyver, A. J., The chemical activities of micro-organisms, London (1931).
5. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **180**, 000 (1949).
6. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **180**, 000 (1949).

THE REDUCTIVE CLEAVAGE OF 4-DIMETHYLAMINOAZO-BENZENE BY RAT LIVER: THE INTRACELLULAR DISTRIBUTION OF THE ENZYME SYSTEM AND ITS REQUIREMENT FOR TRIPHOSPHOPYRIDINE NUCLEOTIDE*

BY GERALD C. MUELLER† AND J. A. MILLER

*(From the McArdle Memorial Laboratory, Medical School,
University of Wisconsin, Madison)*

The hepatic carcinogen, 4-dimethylaminoazobenzene, and its metabolites are subject to at least three metabolic reactions in the intact rat: stepwise demethylation of the dimethylamino group; hydroxylation, principally at the 4' position; and reductive cleavage of the azo linkage to yield monophenylamines (1–3). Although the exact sequence of these reactions and the extent to which each occurs *in vivo* are unknown, it is now possible to study each of these reactions *in vitro* with rat liver homogenates. Thus, in an earlier communication (4) it was reported that freshly prepared rat liver homogenates, fortified with hexose diphosphate, diphosphopyridine nucleotide, and nicotinamide, metabolized 4-dimethyl-aminoazobenzene with formation of small quantities of the demethylated intermediates, 4-monomethylaminoazobenzene and 4-aminoazobenzene, and a new metabolite, 4'-hydroxy-4-dimethylaminoazobenzene. The major reaction, however, appeared to involve a destruction of the azo linkage, since more dye disappeared in these reaction mixtures than could be accounted for by the azo metabolites found.

The present communication deals with the mechanism of the disappearance of the azo dye *in vitro* when incubated with rat liver homogenates. This reaction has been found to consist of the reductive cleavage of the azo linkage in the dye. Furthermore, it has been possible to demonstrate a specific requirement for reduced triphosphopyridine nucleotide as the electron donor for the cleavage reaction and to show the localization of certain components of the enzyme system within the hepatic cell.

Methods

For these experiments triphosphopyridine nucleotide concentrates were prepared by a new procedure developed in this laboratory (5). These

preparations ranged in purity from 10 to 104 per cent, based on Warburg's constant (5.6×10^6 sq. cm. per mole) (6), or from 9 to 95 per cent when calculated by the constant given by Horecker and Kornberg (6.22×10^6 sq. cm. per mole) (7). The triphosphopyridine nucleotide concentrates above 10 per cent purity were considered free of diphosphopyridine nucleotide, since the hydrosulfite assay (8) and the coenzyme-specific *Zwischenferment* assay (5) agreed exactly.

Glucose-6-phosphate dehydrogenase (Warburg's *Zwischenferment*) was prepared essentially according to Warburg and Christian (9) with bottom yeast obtained locally.[1] Glucose-6-phosphate was prepared by the phosphorolysis of starch according to the method of Fantl and Anderson (10), but modified to include a 5 hour hydrolysis of the crude sugar phosphates in 1 N HCl at 100° and followed by the isolation of the ester as the barium salt.

In experiments in which reduced triphosphopyridine nucleotide was used, the dihydro form was generated *in situ* by the addition of an extract of 1.2 mg. of the glucose-6-phosphate dehydrogenase preparation in 0.1 per cent $NaHCO_3$ to the reaction mixture containing the inorganic salts, nicotinamide, glucose-6-phosphate, and the triphosphopyridine nucleotide to be reduced. After incubating the flasks for 30 minutes at 37°, the glucose-6-phosphate dehydrogenase was inactivated by placing the flasks in a boiling water bath for 5 minutes. The flasks were then cooled to 0° and the rat liver preparations added. Control flasks were treated in the same manner, except for the omission of the coenzymes during the incubation period; preparations of oxidized triphosphopyridine nucleotide, oxidized diphosphopyridine nucleotide, and reduced diphosphopyridine nucleotide were then added after the heat inactivation of the glucose-6-phosphate dehydrogenase. Reduced diphosphopyridine nucleotide (71 per cent) was prepared according to the method of Ohlmeyer (11).

The liver samples were obtained from young adult Sprague-Dawley rats maintained on a grain diet and fasted for 18 to 24 hours prior to each experiment. The rats were killed by decapitation and liver samples transferred immediately into ice-cold isotonic KCl. Homogenates (10 to 12 per cent) were prepared in either 8.5 per cent sucrose containing 0.01 M K_2HPO_4-KH_2PO_4 at pH 7.4 or isotonic KCl containing 8.0 ml. of 0.02 M K_2CO_3 per liter, or in distilled water as indicated. All homogenates were prepared at 0°.

In the typical experiment a reaction mixture was prepared which contained the following ingredients adjusted to pH 7.4 in a final volume of 3.0 ml.: 0.2 to 0.4 ml. of homogenate, 0.4 ml. of 0.03 M hexose diphosphate[2]

---

[1] Fauerbach Brewery, Madison, Wisconsin.

[2] Schwarz Laboratories, Inc., New York.

or 0.4 ml. of 0.045 M glucose-6-phosphate, 0.1 ml. of diphosphopyridine nucleotide[3] (DPN) solution (200 γ), 0.1 ml. of triphosphopyridine nucleotide (TPN) solution (200 γ), 0.2 ml. of 0.6 M nicotinamide, 0.1 ml. of 0.1 M $MgCl_2$, 0.4 ml. of 0.5 M KCl, and 0.5 ml. of 0.1 M K_2HPO_4-KH_2PO_4 buffer at pH 7.4. The 4-dimethylaminoazobenzene (50 to 60 γ) was customarily added last in 0.1 ml. of 95 per cent ethanol from a micro blow pipette. The reactions were carried out in open 25 ml. Erlenmeyer flasks with mechanical shaking in a water bath at 37°. In the preliminary experiments TPN was omitted from the reaction mixtures.

After a 30 minute incubation period the reaction was stopped by the addition of 3.0 ml. of 20 per cent trichloroacetic acid in 1:1 acetone-ethanol and then diluted with equal parts of the trichloroacetic acid reagent and water to the appropriate optical density. The precipitated protein was sedimented by light centrifugation and the optical density of the supernatant fluid determined at 520 mμ in a Cenco-Sheard spectrophotelometer adapted to the use of matched 13 mm. $\times$ 100 mm. Pyrex culture tubes. The difference in the optical densities of the zero time control flasks and the incubated samples was used for the calculation of the total dye destruction.

The particulate fractions of the livers were prepared by differential centrifugation of 12 per cent homogenates in 8.5 per cent sucrose solution. The nuclear fraction was sedimented at 600 $\times$ g for 10 minutes in the International multispeed attachment with the No. 295 rotor as described previously (12). The large granules or mitochondria were then sedimented at 18,000 $\times$ g for 10 minutes with the same attachment but with an improved magnalium rotor[4] (13). Each of these fractions was washed with 8.5 per cent sucrose solution and recentrifuged twice; in each case the washings were combined with the supernatant solutions. The small granules or microsomes were obtained by centrifuging the suspension at 120,000 $\times$ g for 35 minutes in an air-driven vacuum ultracentrifuge.[4] The tubes containing the pellets of the small granules were then carefully rinsed with sucrose solution and the washings added to the supernatant solution to form the supernatant fluid fraction. All of the particulate fractions were resuspended in sucrose solution in the plastic centrifuge tubes with Plexiglas pestles and then rehomogenized in all-glass homogenizers if necessary. The suspensions of the particulate fractions were generally prepared at concentrations 4 times that of the original homogenate. Each centrifugation was carried out at 0 to 5° and the relative centrifugal forces were calculated to the centers of the tubes.

[3] The diphosphopyridine nucleotide (79 and 84 per cent) was generously supplied by Dr. G. A. LePage.

[4] Manufactured by Joseph Grebmeier and Sons, Box 235, Menlo Park, California.

Results

Inactivation of Homogenates—10 per cent rat liver homogenates were prepared in distilled water and in isotonic KCl and allowed to stand at 0°. After various intervals tissue samples were pipetted into reaction mixtures fortified with hexose diphosphate, diphosphopyridine nucleotide, and nicotinamide, and assayed for their ability to destroy the dye. The results of a typical experiment are depicted in Fig. 1. With the water homogenate an initial latent period was observed during which activity remained constant; this gave way to a period in which the activity steadily
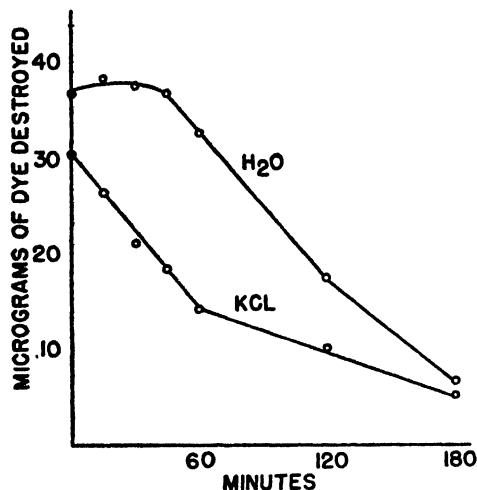
Fig. 1. The inactivation of rat liver homogenates in water and isotonic KCl by storage at 0° for varying periods of time prior to assay for dye destruction activity Except for the omission of triphosphopyridine nucleotide, the system used was as described under "Methods" with 53 γ of dye, 0.3 ml. of 10 per cent rat liver homogenate, and 0.4 ml. of 0.03 M hexose diphosphate in a final volume of 3.0 ml. The results are expressed as micrograms of dye destroyed in 30 minutes.

declined. With KCl homogenates the initial period of constant activity was not observed; however, the rate of decline was approximately equivalent to that of the water homogenate. When the water homogenate was incubated at 37°, the fall in activity was extremely rapid; within 5 minutes the activity was entirely dissipated (Fig. 2).

Reactivation of Homogenates by Triphosphopyridine Nucleotide—Water homogenates which had been inactivated by incubation for 5 minutes at 37° could be reactivated in a number of ways. It was first observed that a 1:1 combination of the inactive homogenate with fresh water homogenate resulted in 15 to 30 per cent reactivation. Thus it appeared that the fresh water homogenate contained an excess of a labile constituent which

was necessary for the destruction of the dye. This labile factor was subsequently demonstrated to be present in heat-inactivated liver homogenates. Certain crude pyridine nucleotide concentrates were also found to contain the factor and it was further noted that the activities of the concentrates were proportional to their content of triphosphopyridine nucleotide. The identity of the factor with triphosphopyridine nucleotide was proved by obtaining complete reactivation with triphosphopyridine nucleotide preparations of 95 to 104 per cent purity (see "Methods"). With hexose diphosphate as the oxidizable substrate complete reactivation was obtained with 50 γ of pure triphosphopyridine nucleotide per flask
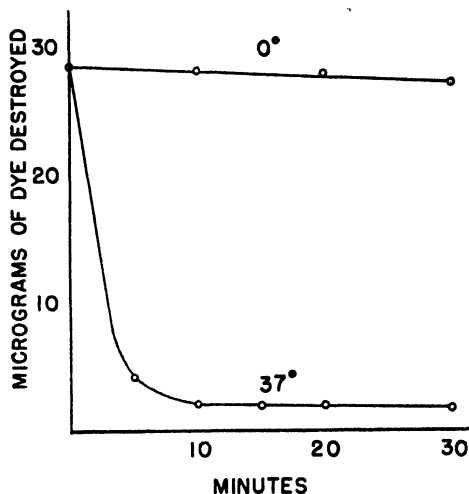
Fig. 2. The inactivation of a rat liver homogenate in water by incubation at 37° for varying periods of time prior to assay for dye destruction activity. The same system as in Fig. 1 was used. The results are expressed as micrograms of dye destroyed in 30 minutes.

(Fig. 3). However, when glucose-6-phosphate was used, 200 to 300 γ of the nucleotide were required (Fig. 3). The increased requirement for triphosphopyridine nucleotide and the smaller amount of dye destruction frequently observed when glucose-6-phosphate was used as the oxidizable substrate instead of hexose diphosphate were not further studied. Glucose-6-phosphate dehydrogenase did not appear to be a limiting factor, since added dehydrogenase (isolated from yeast) failed to stimulate the dye destruction by the whole homogenate. In addition the inactivated whole homogenate required diphosphopyridine nucleotide for reactivation; however, this coenzyme was not required by the isolated small granules and its rôle in the reactions with the whole homogenate has not been elucidated.

Resolution of Enzyme System by Cell Fractionation—From general considerations such as the known fate of the dye *in vivo* and the requirement for triphosphopyridine nucleotide *in vitro*, it appeared probable that the destruction of the dye consisted of a reductive cleavage reaction which required at least two enzyme systems: one which catalyzed the oxidation of a substrate and maintained a reservoir of reduced coenzyme and a second which actually mediated the reductive cleavage of the azo linkage with electrons from the reduced coenzyme. The elucidation of the site of these reactions when glucose-6-phosphate was used as the substrate
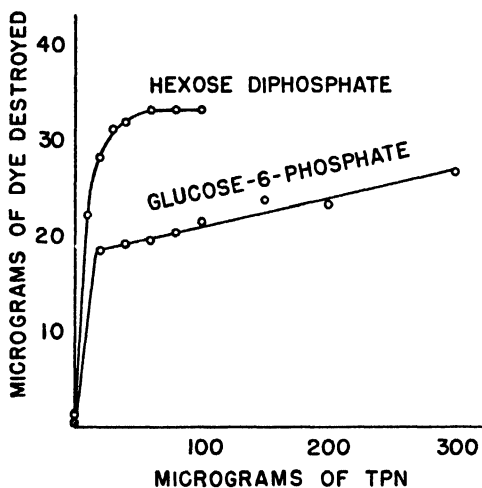
FIG. 3. The reactivation of an incubated rat liver homogenate by triphospho pyridine nucleotide. The system described under "Methods" was used, with varying additions of triphosphopyridine nucleotide (85 per cent assay, DPN-free) to a 3.0 ml. reaction mixture which contained 53 γ of dye, 0.3 ml. of a 10 per cent rat liver homogenate in water (inactivated by incubation for 5 minutes at 37°), and 0.4 ml. of either 0.03 M hexose diphosphate or 0.045 M glucose-6-phosphate as indicated. The results are expressed as micrograms of dye destroyed in 20 minutes.

was greatly facilitated by the use of differential centrifugation for the separation of liver homogenates in isotonic sucrose solution into four fractions: a nuclear fraction, the large granules or mitochondria, the small granules or microsomes, and the supernatant fluid. When the liver fractions were assayed separately for their abilities to destroy dye, they were all essentially inactive; however, the combination of the supernatant fluid with any particulate fraction resulted in the destruction of dye (Fig. 4). The largest activation was observed with the small granules which account for only 12 per cent of the protein in the rat liver (12). The inactivity of the combination of the nuclei, large granules, and small granules in-

dicated that a glucose-6-phosphate dehydrogenase was present in the supernatant fluid. It has been possible to demonstrate this directly[5] by an adaptation of the *Zwischenferment* assay and it was further substantiated by the effective substitution of yeast glucose-6-phosphate dehydrogenase for the supernatant fluid from liver. Hence it appeared that, when glucose-6-phosphate was used as the oxidizable substrate, the function of the supernatant fluid was to maintain a reservoir of reduced coenzyme and the enzyme responsible for the destruction of the dye resided in the particulate fractions.
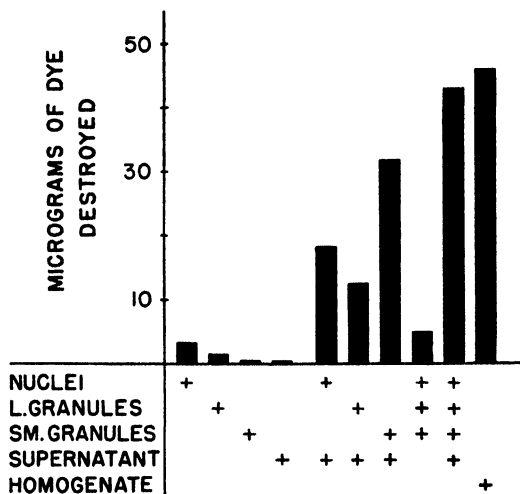
FIG. 4. The destruction of 4-dimethylaminoazobenzene by intracellular fractions isolated from rat liver by differential centrifugation. The systems described under "Methods" were used, with 53 γ of dye and 0.4 ml. of 0.045 M glucose-6-phosphate per 3.0 ml. of reaction mixture. Intracellular fractions were added to the reaction mixture in amounts equivalent to that derived from 50 mg. of whole rat liver. The results are expressed as micrograms of dye destroyed in 30 minutes.

When hexose diphosphate was used instead of glucose-6-phosphate as the oxidizable substrate with the cell fractions, no sharp separation of the enzyme activity into two mutually dependent systems was evident.

Coenzyme Specificity of Enzyme System—The finding that the greatest concentration of the dye destruction activity resided in the small granules which could be easily freed of glucose-6-phosphate dehydrogenase activity by high speed centrifugation provided a convenient tool for the demonstration of the absolute coenzyme specificity of the enzyme system catalyzing the destruction of the dye. In this experiment, isolated small granules

[5] G. C. Mueller and J. A. Miller, unpublished data.

were incubated with 4-dimethylaminoazobenzene in reaction mixtures containing 1.0 mg. samples of the oxidized and reduced forms of the two pyridine coenzymes, as indicated in Fig. 5. The oxidized forms of diphosphopyridine and triphosphopyridine nucleotide were inactive in the system and served to show the absence of any significant quantity of endogenous electron donors. However, when the reduced coenzymes were added as the sole source of electrons, only the addition of dihydrotriphosphopy-

SM. GRANULES		+	+	+	+	+	+	+
TPN			+	+	+			
TPN·H₂	+						+	+
DPN				+	+			+
DPN·H₂		+			+	+		
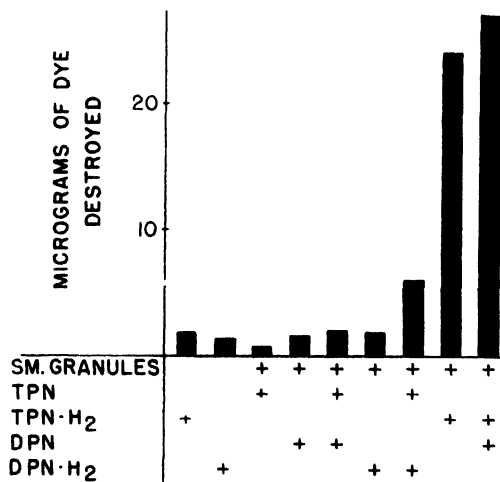
Fig 5. The requirement for reduced triphosphopyridine nucleotide by the azo dye-cleaving enzyme system in the small granules of rat liver. 1 mg. samples of diphosphopyridine and triphosphopyridine nucleotide were added to reaction flasks containing the small granules from 100 mg. of whole rat liver, 0 4 ml. of 0.045 M glucose-6-phosphate, 0.5 ml. of 0.1 M KH₂PO₄-K₂HPO₄ buffer at pH 7.4, 0.4 ml. of 0.5 M KCl, 0.1 ml of 0.1 M MgCl₂, 0.2 ml. of 0.6 M nicotinamide, 0 2 ml. of heat-inactivated glucose-6-phosphate dehydrogenase from yeast, and 53 γ of 4-dimethylaminoazobenzene Reduced triphosphopyridine nucleotide was formed in the reaction flasks as described under "Methods." The results are expressed as micrograms of dye destroyed in a 30 minute incubation period The oxidized triphosphopyridine nucleotide, oxidized diphosphopyridine nucleotide, and reduced diphosphopyridine nucleotide were preparations of 71 to 79 per cent purity.

ridine nucleotide was effective in promoting the destruction of dye. The small amount of activity observed with dihydrodiphosphopyridine nucleotide when combined with oxidized triphosphopyridine nucleotide suggested that an electron transfer mechanism between the two coenzymes may have been present.

Isolation of Cleavage Products of Azo Dye—A search was made for any monophenylamines formed upon the destruction of the azo dye in these reaction mixtures, since a method had become available for the separation

and determination of the six monophenylamines that might be formed from this dye (14). In essence the Böniger reaction is used in which primary amines and sodium β-naphthoquinone sulfonate react at pH 7.0 to form colored Schiff bases which may be differentiated on the basis of their absorption spectra and solubilities in various solvents. In one of the experiments 53 γ of 4-dimethylaminoazobenzene were incubated in a reaction mixture containing the small granules from 300 mg. of rat liver, the supernatant fluid from 83 mg. of rat liver, 395 γ of triphosphopyridine nucleotide, 500 γ of diphosphopyridine nucleotide, 0.4 ml. of 0.045 M glucose-6-phosphate, 0.2 ml. of 0.6 M nicotinamide, 0.5 ml. of 0.1 M K phosphate buffer at pH 7.4, 0.4 ml. of 0.5 M KCl, 0.1 ml. of 0.1 M MgCl$_2$, and water to a final volume of 4.0 ml. The reaction was conducted in an atmosphere of nitrogen at 37° for 30 minutes. After this time no residual dye was demonstrable in the reaction mixture.

For the recovery of steam-volatile amines such as aniline, 0.1 ml. of 50 per cent KOH was added to the reaction flask and the contents of the flask were transferred to a micro steam distillation apparatus. A few mg. of octadecyl alcohol were added to control the foaming and the mixture was then distilled for 20 minutes. The distillate was collected in 3.0 ml. of 0.1 per cent sodium β-naphthoquinone sulfonate in 1.0 M phosphate buffer at pH 7.0, and this solution was then extracted with 4.0 ml. of benzene. A yellow benzene solution was obtained which gave the characteristic absorption curve of the Schiff base of aniline with a maximum at 450 mμ. From the optical density it was calculated that 22 γ of aniline were recovered; this represented 100 per cent of the theoretical amount of aniline that could be formed from the amount of dye added.

The formation of N,N-dimethyl-p-phenylenediamine in an identical reaction mixture was also demonstrated by the isolation of the Schiff base of this amine. At the termination of the incubation period 0.1 ml. of 50 per cent H$_2$SO$_4$ was added to the reaction flask and the precipitated proteins sedimented by centrifugation. The supernatant fluid and a 3.0 ml. wash of the precipitate were transferred to a 50 ml. Erlenmeyer flask containing 1 drop of tributyl phosphate[6] as an antifoam agent. After neutralization with solid NaHCO$_3$ to pH 7.0, 3.0 ml. of the β-naphthoquinone reagent were added and the solution extracted with 4.0 ml. of benzene. A blue purple benzene solution was obtained; the absorption curve of this solution had a maximum at 580 mμ and otherwise agreed with that observed for the Schiff base of N,N-dimethyl-p-phenylenediamine. The addition of acetic anhydride to the benzene solution produced only a slight decrease in optical density and indicated that less than 10 per cent of the color could be accounted for as the Schiff base of N-monomethyl-p-

phenylenediamine. Thus from the optical density it was calculated that 16.6 γ of N,N-dimethyl-p-phenylenediamine were recovered from the reaction mixture, or approximately 50 per cent of the theoretical yield. The relatively low recovery of this amine was not unexpected, since it is readily oxidized.

When the monophenylamines were recovered from whole homogenates shaken with the dye in an atmosphere of air, approximately 70 per cent of the theoretical yield of aniline and 30 per cent of the theoretical yield of the methylated phenylenediamines were recovered in this manner. These findings demonstrated that reductive cleavage of the azo linkage accounted for the majority of the destruction of dye under these experimental conditions.

No p-phenylenediamine or p-aminophenol was detected after the dye had been destroyed by the homogenates or small granules. These amines could result from the reductive cleavage of certain of the azo metabolites of the added dye (4).

DISCUSSION

Under the conditions of these experiments the reaction which was mainly responsible for the destruction of 4-dimethylaminoazobenzene by the rat liver preparations was shown to consist of the reductive cleavage of the azo linkage in this dye to yield the corresponding monophenylamines. Furthermore, this reaction was shown to be catalyzed by an enzyme system which specifically required reduced triphosphopyridine nucleotide for mediation of the reductive cleavage. When glucose-6-phosphate was utilized as the oxidizable substrate, the cleavage reaction could be separated by differential centrifugation into two parts (Fig 6.), an electron-donating system and the azo dye-cleaving system. The specific dehydrogenase was found to be confined to the supernatant fluid where it catalyzed the oxidation of the substrate and maintained a reservoir of reduced triphosphopyridine nucleotide. The particulate fractions contained the enzyme or enzymes which catalyzed the reductive cleavage of the azo linkage in the dye with electrons from the reduced nucleotide. These enzymes were relatively highly concentrated in the small granules and these particles could catalyze the reduction of the dye in the presence of reduced triphosphopyridine nucleotide as the sole electron donor. The whole homogenate also required the presence of diphosphopyridine nucleotide for reactivation, but the rôle of this coenzyme in the cleavage reaction is unknown; the experiments with isolated small granules failed to show a requirement of this factor.

The rôle that reductive cleavage of 4-dimethylaminoazobenzene plays in the carcinogenic process induced in the liver by this dye is not known;

it may be simply a means of detoxication. However, it is of interest in this connection that preliminary studies[6] have shown that the rat liver, the sole site of carcinogenic action by the dye, was the most active tissue in causing the destruction of dye *in vitro;* kidney was about 30 per cent as active and spleen, lung, heart, intestine, and brain were essentially inactive. Although in several studies (15–17) the free amines produced by the reductive cleavage of 4-dimethylaminoazobenzene did not exhibit carcinogenic activity in the rat when included in the diet at high levels, White, Eschenbrenner, and White (18) reported in an abstract that 10 per cent of rats fed aniline for 14 to 31 months developed hepatomas.
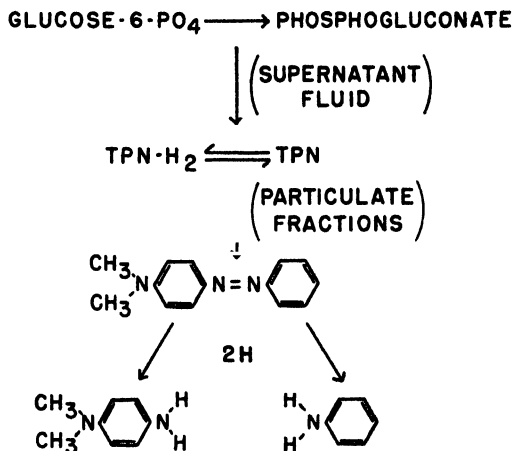
FIG. 6. The reductive cleavage of 4-dimethylaminoazobenzene by the supernatant fluid and particulate fractions of rat liver.

Since cirrhosis and low incidences of hepatomas can be induced by dietary means (19–21), interpretation of this result will have to wait until the data have been published in full. However, while there is no evidence that aniline or N,N-dimethyl-p-phenylenediamine is the causal agent in carcinogenesis by 4-dimethylaminoazobenzene, the data presented in this paper, indicating that these amines could and may well be generated from the dye in the intact liver, provide an interesting approach to the study of azo dye carcinogenesis.

SUMMARY

Rat liver homogenates fortified with glucose-6-phosphate, diphosphopyridine nucleotide, triphosphopyridine nucleotide, and nicotinamide catalyzed the reductive cleavage of the hepatic carcinogen 4-dimethylaminoazobenzene to yield the corresponding monophenylamines, N,N-dimethyl-

p-phenylenediamine and aniline. The cleavage reaction was shown to be composed of at least two enzyme systems: one enzyme system which constituted a source of reduced triphosphopyridine nucleotide through the oxidation of glucose-6-phosphate and a second enzyme system which specifically required reduced triphosphopyridine nucleotide for the reductive cleavage of the azo dye. By differential centrifugation the former system was shown to be present entirely in the supernatant fluid remaining after removal of the particulate fractions from the homogenates; the latter system resided entirely in the particulate fractions, with the highest concentration being found in the small granules. The small granules catalyzed the reduction of the dye in the presence of reduced triphosphopyridine nucleotide as the sole electron donor; the whole homogenate required in addition diphosphopyridine nucleotide.

BIBLIOGRAPHY

1. Miller, J. A., Miller, E. C., and Baumann, C. A., *Cancer Res.*, **5**, 162 (1945).
2. Miller, J. A., and Miller, E. C., *Cancer Res.*, **7**, 39 (1947).
3. Stevenson, E. S., Dobriner, K., and Rhoads, C. P., *Cancer Res.*, **2**, 160 (1942)
4. Mueller, G. C., and Miller, J. A., *J. Biol. Chem.*, **176**, 535 (1948).
5. LePage, G. A., and Mueller, G. C., *J. Biol. Chem.*, **180**, 975 (1949).
6. Warburg, O., *Ergeb. Enzymforsch.*, **7**, 210 (1938).
7. Horecker, B. L., and Kornberg, A., *J. Biol. Chem.*, **175**, 385 (1948).
8. LePage, G. A., *J. Biol. Chem.*, **168**, 623 (1947).
9. Warburg, O., and Christian, W., *Biochem. Z.*, **254**, 438 (1932).
10. Fantl, P., and Anderson, C. M., *Australian J. Exp. Biol. and Med. Sc.*, **19**, 117 (1941).
11. Ohlmeyer, P., *Biochem. Z.*, **297**, 66 (1938).
12. Price, J. M., Miller, E. C., and Miller, J. A., *J. Biol. Chem.*, **173**, 345 (1948).
13. Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., *Cancer Res.*, **9**, 96 (1949).
14. Miller, E. C., Miller, J. A., Sapp, R. W., and Weber, G. M., *Cancer Res.*, **9**, 336 (1949).
15. Miller, J. A., and Baumann, C. A., *Cancer Res.*, **5**, 227 (1945).
16. Sugiura, K., Halter, C. R., Kensler, C. J., and Rhoads, C. P., *Cancer Res.*, **5**, 235 (1945).
17. Miller, J. A., and Miller, E. C., *J. Exp. Med.*, **87**, 139 (1948).
18. White, F. R., Eschenbrenner, A. B., and White, J., Abstracts, 4th International Cancer Congress, St. Louis, 75 (1947).
19. Webster, G. T., *J. Clin. Invest.*, **21**, 385 (1942).
20. Copeland, D. H., and Salmon, W. D., *Am. J. Path.*, **22**, 1059 (1946).
21. Engel, R. W., Copeland, D. H., and Salmon, W. D., *Ann. New York Acad. Sc.*, **49**, 49 (1947).

THIAMINE TRIPHOSPHORIC ACID. ITS RELATION TO THE THIAMINE PYROPHOSPHATE (COCARBOXYLASE) OF LOHMANN AND SCHUSTER

By LÉON VELLUZ, GASTON AMIARD, and JAROSLAV BARTOS

(*From the Chemical Department, Roussel Laboratories, Paris, France*)

In previous publications (18, 19) the preparation of the triphosphoric. ester of thiamine, $C_{12}H_{19}O_{10}N_4SP_3 \cdot \frac{1}{2}H_2O$, has been reported. In this substance 2 of the 3 phosphorus atoms hydrolyze in acid media; the 3rd is cleaved only by alkaline saponification. The analytical data agree well with the following formula.
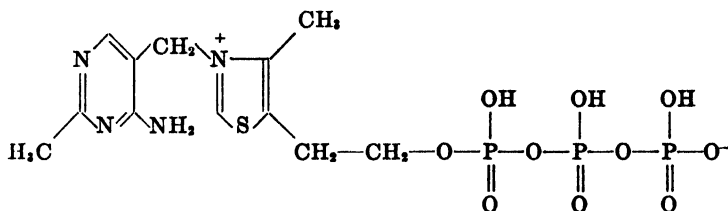
By analogy with the designation ATP for adenosine triphosphoric acid, TTP is proposed for the new triphosphoric acid derivative of thiamine. The most significant physiological properties of TTP are concerned with the heart (16, 17, 21), TTP being much more active than thiamine or cocarboxylase.

Investigations in the enzymatic field have now been made in order to compare TTP with cocarboxylase and to determine the activity of potato apyrase and adenosine triphosphatase (ATPase) towards TTP.

EXPERIMENTAL

Washing of Yeast—Most authors (15, 26, 27) have followed with some modifications the washing procedure described by Lohmann and Schuster (7, 8); the latter as well as Liebknecht (5) consider that bakers' yeast contains from 10 to 20 times less carboxylase than brewers' yeast. The procedure of Westenbrink *et al.* (27) has been used with bakers' yeast and has led to satisfactory results. Bakers' yeast, dried by exposure to air for several days, was crushed in a mortar, sifted, and kept in a tightly stoppered flask; it could be used for a great number of experiments.

A suspension of 1 gm. of dry yeast in 20 cc. of water was centrifuged for 3 minutes and the supernatant discarded even if turbid. Further centrif-

ugations were carried out in the same way. To the residue were successively added 20 cc. of a half saturated ammonium sulfate solution and 20 cc. of phosphate buffer, pH 10. After 5 minutes of vigorous shaking, the mixture was centrifuged; 20 cc. of water and 20 cc. of phosphate buffer, pH 10, were added to the residue. After 4 minutes shaking the mixture was centrifuged, 40 cc. of water were added to the residue, and the shaking repeated. As soon as the suspension became homogeneous, it was centrifuged for 2 minutes and the residue washed twice again with water as rapidly as possible.[1]

Reconstitution of Cocarboxylase—Various authors (1, 7, 8) have established that any bivalent ion could be used instead of Mg^{++} for the reconstitution of the enzyme system; Mn^{++}, which is the most active and according to Kossel (3) would protect carboxylase against inhibitors, was used in our experiments, as has been proposed by Westenbrink and Parve (15, 26, 27). It is known that thiamine is also required in the reactive medium and it seems to be proved (5, 6, 12, 13, 24–26) that thiamine inhibits a yeast phosphatase capable of hydrolyzing cocarboxylase only when not linked to the specific protein. Our results are in agreement with this point of view; moreover chlorothiamine was found to exert a similar activity.

The washed product obtained above was suspended in 0.02 M manganese chloride solution, in such quantity that 1 cc. contained 0.20 gm. of the initial dry yeast. All further manipulations were carried out in calibrated 10 cc. glass-stoppered flasks.

To these flasks were added 1 cc. of the homogenous yeast suspension and 0.2 cc. of a 10^{-4} M solution of thiamine hydrochloride (20 γ). The substance to be tested for cocarboxylase activity was separately dissolved in 1 cc. of phosphate buffer, pH 6.8.

After 15 minutes shaking in a water bath at 28°, the reconstitution of the enzyme system was achieved. The suspension was centrifuged during 6 minutes and the clear supernatant discarded; to the residue were added 15 cc. of acetate buffer, pH 5.7, and 5 cc. of a 2 per cent sodium pyruvate solution. The stoppered flask was heated at 28° in a water bath and shaken throughout the entire fermentation.

Determination of Cocarboxylase Activity—The activity of cocarboxylase is generally tested by measurement of the quantity of CO_2 evolved from pyruvate in the Warburg apparatus. Acetoin is produced during this reaction (11), but the amount is so small that it can be neglected (4, 7, 8); by the Voges-Proskauer reaction (14) it was less than 5 per cent both with TTP and cocarboxylase.

[1] Except when otherwise stated, every centrifugation lasted 3 minutes at 3400 R.P.M.

In some cases fluorometric titration of acetaldehyde was used instead of measurement of the evolved CO_2. The method devised in this laboratory (20, 22, 23) is based upon the condensation of acetaldehyde with 3,5-diaminobenzoic acid.

At the end of the fermentation the flasks were chilled in ice for 5 minutes. Their content was adjusted to 10 cc. by addition of ice-cold water. The flasks were stoppered at once and centrifuged. This procedure decreased the loss of acetaldehyde and gave reproducible results.

Amounts of 0.5 to 2 cc. of the supernatant were pipetted out so that the sample contained about 40 to 100 γ of acetaldehyde. The titration
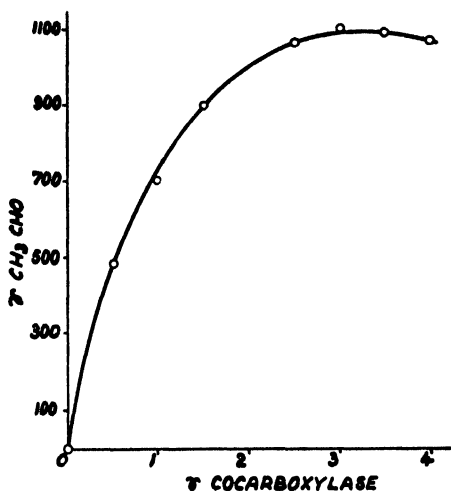
FIG. 1. Acetaldehyde produced as a function of the weight of cocarboxylase added to a constant weight of washed yeast (corresponding to 0.2 gm. of dry yeast). Time of fermentation 15 minutes; temperature 28°. .

was then carried out as described previously (23). The results were in fair agreement with those given by the Warburg manometer.

Comparison between TTP and Cocarboxylase

The acetaldehyde produced as a function of the amount of cocarboxylase or TTP added to a constant weight of bakers' yeast is shown in Fig. 1.

The volumes of CO_2 calculated from the amount of acetaldehyde were higher than those published by Lohmann and Schuster for bottom brewers' yeast, but we are in good agreement with the results of Westenbrink, Parve, van der Linden, and van den Broek (27). These authors, by adding 0.1 γ of cocarboxylase to 200 mg. of dry brewers' yeast, obtained about 100 c.mm. of CO_2 in 30 minutes. In Fig. 2, 0.1 γ added to 200 mg. of

dry bakers' yeast corresponds to about 100 γ of acetaldehyde, *i.e.* 50 c.mm. of CO_2 in 15 minutes; the blanks were negative. Our technique of washing bakers' yeast thus seems satisfactory.[2]

For TTP and cocarboxylase the curves have the same general aspect, but for TTP the plateau appeared only beyond the 15 γ dose. About 4 to 5 times more TTP than cocarboxylase were required for saturation. The enzymatic system built up with TTP had only 80 per cent of the activity of the system containing cocarboxylase.

With brewers' yeast the results obtained with the Warburg apparatus (Fig. 3) show that TTP has from 80 to 90 per cent of the activity of cocarboxylase.

Lohmann and Schuster (7, 8) have pointed out that in the case of cocarboxylase the amount of CO_2 increases proportionally with time. This is true only at the beginning of the fermentation; afterward the acetaldehyde
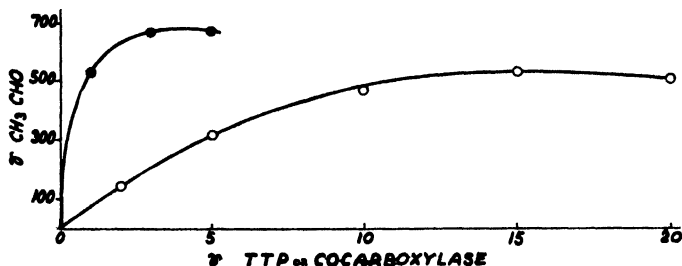
FIG. 2. Acetaldehyde produced as a function of the weight of cocarboxylase or of TTP added to a constant weight of washed yeast (corresponding to 0.2 gm. of dry yeast). Time of fermentation 15 minutes; temperature 28°. ○ TTP; ● cocarboxylase.

inhibits the reaction (Fig. 4). This was also observed with TTP, but the reaction was less intense, perhaps because less acetaldehyde was formed.

CO_2 Formed by Simultaneous Action of TTP and Cocarboxylase on Brewers' Yeast—Table I shows that in the range in which the yeast is not saturated by addition of 1 γ of cocarboxylase the gradual addition of TTP (from 0 to 15 γ) enhances the activity of the resynthesized compound. Enzymatic activity rises to a value greater than the sum of the separate activities of cocarboxylase and of TTP in equivalent amount.

This enhancement of cocarboxylase activity was calculated by the formula

$$E = \frac{(CO_2 \text{ measured}) - (CO_2 \text{ calculated})}{CO_2 \text{ calculated}}$$

[2] In order to get reproducible results, it is essential to perform all titrations simultaneously. Slight differences in working conditions entail important changes in the activity of the reconstituted ferment. In this respect comparison of Figs. 1 and 2 reveals marked differences from one assay to another.
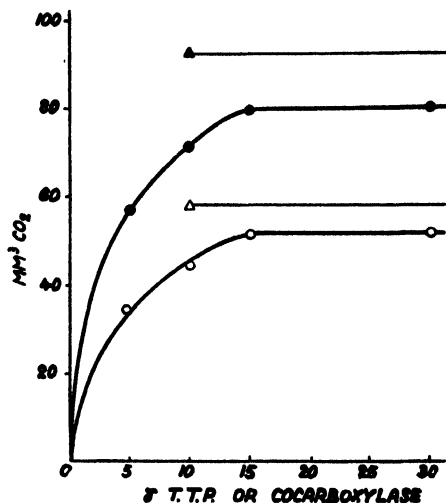
Fig. 3. CO_2 produced as a function of the weights of cocarboxylase or of TTP. Weight of brewers' yeast, 0.1 gm.; time of fermentation 10 and 15 minutes; temperature 28°. The cocarboxylase is represented by a single point, which has been chosen in the range of saturation and indicates the plateau. O TTP, time of fermentation 10 minutes; ● TTP, time of fermentation 15 minutes; △ cocarboxylase, time of fermentation 10 minutes; ▲ cocarboxylase, time of fermentation 15 minutes.
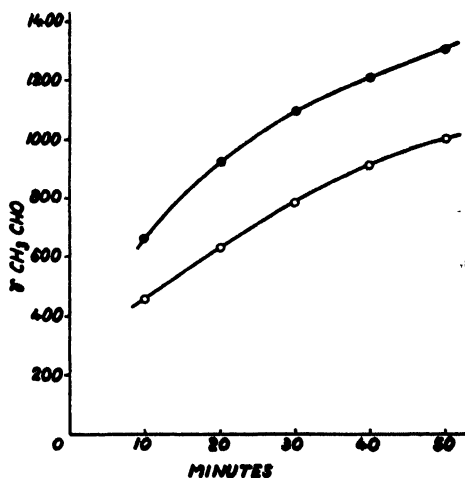
Fig. 4. Acetaldehyde produced as a function of the time for 0.2 gm. of initial dry yeast (bakers' yeast saturated with cocarboxylase or with TTP). O TTP, 20 γ; ● cocarboxylase, 4 γ.

The CO_2 measured equals the quantity of CO_2 formed in the Warburg manometer with the simultaneous action of 1 γ of cocarboxylase and in-

creasing quantities of TTP. The CO_2 calculated equals the sum of the amounts of CO_2 evolved in the Warburg apparatus after separate experiments with TTP and cocarboxylase (Fig. 5).

TABLE I

Activity of TTP in Presence of Cocarboxylase

TTP	CO₂ produced, c mm.					
	In 10 min.			In 20 min.		
	Cocarboxylase, 0	Cocarboxylase, 1 γ	Cocarboxylase, 1 γ + TTP	Cocarboxylase, 0	Cocarboxylase, 1 γ	Cocarboxylase, 1 γ + TTP
γ						
0	0	25	25	0	50	50
1	4	36	29	9	67	59
4	13	52	38	27	100	77
10	35	75	60	67	141	117
15	52		77	101	167	151

Quantity of CO_2 measured in the Warburg manometer in c.mm. per 100 mg. of brewers' yeast. The columns "Cocarboxylase, 1γ + TTP" indicate the quantities which would have been obtained if the activities of 1γ of cocarboxylase and of TTP were added arithmetically.
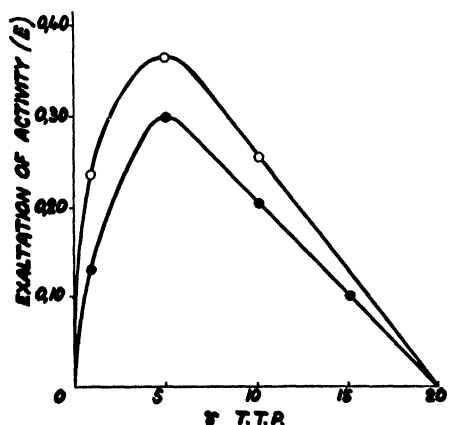
FIG. 5. Increase of activity (E) of 1γ of cocarboxylase by addition of TTP. ○ time of fermentation 10 minutes; ● time of fermentation 20 minutes.

The increase is maximum for 4γ of TTP and 1γ of cocarboxylase. By extrapolation it seems that it should be nil for about 20γ of TTP; *i.e.*, roughly the saturating amount of TTP towards the yeast.

In the range in which the yeast is normally saturated with cocarboxylase (5γ), the total activity is near the sum of the separate activities of cocar-

boxylase and of TTP in corresponding quantities (Table II). The value found for 20 γ of TTP and 5 γ of cocarboxylase after 20 minutes of fermentation (212 c.mm.) must be considered suspicious, the accuracy of the results decreasing above 150 c.mm.

Action of Potato Apyrase and Muscle ATPase on TTP

According to Kalckar (2) and Meyerhof (9) potato apyrase liberates two phosphoric groups from ATP, the first faster than the second.

In a first step, the action of apyrase on TTP should yield a certain proportion of cocarboxylase, and then, in a second step, thiamine orthophosphoric acid. Thus, only the first step should yield hydrolysis products

TABLE II

Activity of TTP in Presence of Cocarboxylase

	CO_2 produced, c.mm.					
	In 10 min.			In 20 min.		
TTP	Cocarboxylase, 0	Cocarboxylase, 5 γ	Cocarboxylase, 5 γ + TTP	Cocarboxylase, 0	Cocarboxylase, 5 γ	Cocarboxylase, 5 γ + TTP
γ						
0	0	42	42	0	79	79
5	16	62	58	39	128	118
10	37	80	79	72	152	151
20	62	100	104	108	212	187

Quantity of CO_2 measured with the Warburg apparatus in c.mm. per 100 mg. of brewers' yeast. The columns "Cocarboxylase, 5 γ + TTP" indicate the quantities which would be obtained if the activities of 5 γ of cocarboxylase and of TTP were added arithmetically.

which will be active in the decarboxylation of pyruvic acid. However, muscle ATPase, if it has any action on TTP, should liberate only cocarboxylase, which should be more active throughout than the initial substance.

Apyrase—Apyrase was prepared by extracting potato pulp with an equal weight of water. The extract was precipitated by 2 volumes of acetone at −10° and the precipitate redissolved in the minimum amount of water. The solution was filtered and solid ammonium sulfate added to 60 per cent of saturation. After filtration the solution was saturated with ammonium sulfate and allowed to stand overnight at 20°. The precipitate was collected and redissolved in water (100 cc. per kilo of the initial weight of potato). The final enzymatic solution contained about 2 mg. of protein per cc.

ATPase was prepared from guinea pig muscle according to Mommaerts and Seraidarian (10).

TTP Hydrolysis—To 20 mg. of TTP were added 1 mg. of enzymatic protein and 0.5 M acetate buffer (pH 6.5) to a final volume of 5 cc. In the case of ATPase, KCl (0.5 M) and CaCl₂ (0.01 M) were added to the medium. In the blanks the enzyme was heated for 15 minutes at 100°.

After 15 to 120 minutes at 37°, 0.6 cc. of the reaction mixture was diluted to 50 cc. From this solution 0.1 cc., corresponding to 4.8 γ of the initial TTP, was pipetted for titration with the Warburg manometer.

Table III shows that after 15 minutes apyrase liberates a compound more active in the decarboxylation of pyruvic acid than the starting substance. After 120 minutes hydrolysis the product was less active than TTP. Therefore it is obvious that, in a first step, apyrase liberates a

TABLE III

Action of Apyrase on TTP

Preparation of apyrase	After 15 min. hydrolysis			After 120 min. hydrolysis		
	Length of time of decarboxylation					
	10 min.	20 min.	30 min.	5 min.	10 min.	15 min.
1	57	124	171	24	46	76
1 (Inactivated)	33	71	90	31	59	90
2	56	107	172	36	76	111
2 (Inactivated)	39	80	119	44	87	140

The figures given are the average of two measurements in the Warburg manometer.

product which, like cocarboxylase, is more active than TTP, while prolongation of hydrolysis leads to a mixture less active than the initial product. The residual activity was due to the unsplit compounds.

The potato apyrase thus hydrolyzes TTP as well as ATP. These data provide enzymatic evidence of the triphosphoric structure of TTP.

While ATPase acted on ATP, it was quite ineffective towards TTP. Thus ATPase is strictly specific for ATP and devoid of any hydrolyzing activity on TTP.

SUMMARY

1. Thiamine triphosphoric acid (TTP), recently described by the authors, restores the carboxylase activity of washed yeast. From 4 to 5 times more TTP than cocarboxylase are required to saturate the washed yeast. The resynthesized enzymatic system developed 80 per cent of the activity of the one rebuilt with cocarboxylase. In the course of the study a new

washing technique for bakers' yeast and a titration method for the acetaldehyde formed from pyruvate are described.

2. Potato apyrase hydrolyzes TTP in two steps to pyrophosphoric and to orthophosphoric acid ester. ATPase is devoid of any hydrolyzing activity towards TTP.

3. The difference in activity of TTP and cocarboxylase indicates that TTP does not act solely through a pyrophosphoric ester such as cocarboxylase due to its hydrolysis.

BIBLIOGRAPHY

1. Green, D. E., Herbert, D., and Subrahmanyan, V., J. Biol. Chem., 135, 795 (1940).
2. Kalckar, H. M., J. Biol. Chem., 153, 355 (1944).
3. Kossel, A. J., Z. physiol. Chem., 276, 251 (1942).
4. Langenbeck, W., Wrede, H., and Schlockermann, W., Z. physiol. Chem., 227, 263 (1934).
5. Liebknecht, W L., Biochem. Z., 303, 101 (1939–40).
6. Lipton, M. A., and Elvehjem, C. A , J. Biol. Chem., 136, 637 (1940).
7. Lohmann, K., and Schuster, P., Naturwissenschaften, 25, 26 (1937).
8. Lohmann, K., and Schuster, P., Biochem. Z., 294, 188 (1937).
9. Meyerhof, O., J. Biol. Chem., 157, 105 (1945).
10. Mommaerts, W. F. H. M., and Seraidarian, K., J. Gen. Physiol., 30, 401 (1947).
11. Neuberg, C., and Hirsch, J., Biochem. Z., 115, 282 (1921).
12. Ochoa, S., Nature, 141, 831 (1938).
13. Ochoa, S., and Peters, R. A., Biochem. J., 32, 1501 (1938).
14. O'Meara, R. A. Q., J. Path. and Bact., 34, 401 (1931).
15. Parve, E. P. S., and Westenbrink, H. G. K., Z. Vitaminforsch., 15, 1 (1944).
16. Plotka, C , and Jequier, R., Compt. rend. Soc. biol , 142, 727 (1948).
17. Plotka, C., and Peterfalvi, M., Compt rend. Soc. biol., 142, 836 (1948).
18. Velluz, L., Amiard, G., and Bartos, J., Compt. rend. Acad., 226, 735 (1948).
19. Velluz, L., Amiard, G., and Bartos, J , Bull. Soc. chim., 15, 871 (1948).
20. Velluz, L., Amiard, G., and Pesez, M., Bull. Soc. chim., 15, 678 (1948).
21. Velluz, L., Jequier, R., and Plotka, C., Compt. rend. Acad., 226, 1855 (1948).
22 Velluz, L., Pesez, M., and Amiard, G., Bull. Soc. chim., 15, 680 (1948).
23. Velluz, L., Pesez, M., and Herbain, M., Bull. Soc. chim., 15, 681 (1948).
24. Westenbrink, H. G. K., and van Dorp, D. A., Nature, 145, 465 (1940).
25. Westenbrink, H. G. K., van Dorp, D. A., Gruber, M., and Veldmann, H., Enzymologia, 9, 73 (1940).
26. Westenbrink, H. G. K , and Parve, E. P. S., Rec. trav. chim. Pays-Bas, 61, 146 (1942).
27. Westenbrink, H. G. K., Parve, E. P. S., van der Linden, A. C., and van den Broek, W. A., Z. Vitaminforsch., 13, 218 (1943).

FACTORS INFLUENCING THE ELECTROPHORETIC ANALYSIS OF HUMAN SERUM

By DAN H. MOORE, J. B. ROBERTS, M. COSTELLO, AND
T. W. SCHONBERGER

*(From the Electrophoresis Laboratory, College of Physicians and Surgeons,
Columbia University, New York)*

The question has often arisen as to how serum or plasma should be obtained and handled preparatory to electrophoretic analysis. Alterations in the electrophoretic pattern of plasma and plasma fractions resulting from prolonged storage and various treatments have already been reported (1–4). It has been observed, however, that changes may occur in a short period of time under apparently normal conditions if certain precautions are not observed. The effect of hemolysis has been quantitatively measured. In phosphate buffer, the γ-globulin is affected, whereas in veronal buffer the β-globulin is affected. The changes in the serum or plasma which occur on standing appear to depend on two factors, lipides and proteins. Those depending on the lipides or lipoproteins occur more readily than those dependent on the proteins alone.

This paper reports alterations in the electrophoretic pattern caused by (1) hemolysis, (2) dialysis, and (3) short periods of storage or shipping. It further reports on the accuracy and reproducibility of pattern measurements.

Method

The samples of sera and the methods of their preparation will be described in detail below. Two types of buffer solutions were employed: one buffer (5, 6) of pH 7.4 was 0.02 M with respect to sodium phosphate and 0.15 M with respect to NaCl (referred to as phosphate buffer) and the other was a veronal buffer of pH 8.6 which was 0.1 M with respect to sodium diethyl barbiturate and 0.02 M with respect to diethylbarbituric acid (7) (referred to as barbiturate or veronal buffer). Each sample, unless otherwise stated, was dialyzed for 24 hours. All of the analyses were made in a Tiselius micro cell of 2 cc. capacity (8, 9). Some of the photographic records were obtained by the scanning method of Longsworth (10) and others were obtained with a slight modification of this method (11). Each pattern was projected at a magnification of 3.5 times, traced and measured with a planimeter. The averages taken from three to five tracings are recorded in Tables I to IV.

Hemolysis—It is quite evident that since hemoglobin is a protein, which is isoelectric at about pH 7.0, hemolysis will cause an increase in one of the electrophoretic components of serum. In routine practice some hemolysis very often occurs in the drawing or handling of blood. Here an attempt has been made to determine the extent of hemolysis necessary to cause a measurable change in the electrophoretic pattern of the sera and to correlate the hemoglobin content as measured both by nitrogen and light absorption with its effect on pattern area.
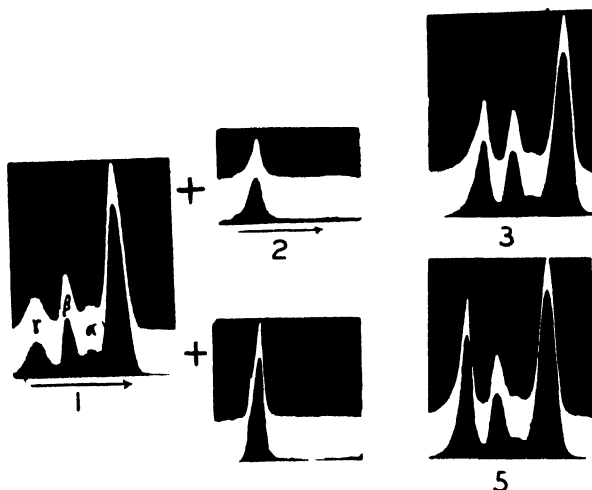
FIG. 1. Electrophoretic patterns of the same serum showing the effect of hemolysis in phosphate buffer. (*1*) non-hemolyzed serum, (*2*) 0.39 per cent hemoglobin, (*3*) hemolyzed serum, (*4*) 0.78 per cent hemoglobin, (*5*) hemolyzed serum.

Sample I—A sample of non-hemolyzed serum was electrophoretically analyzed in a phosphate buffer, then again after specific quantities of hemoglobin were added (see Fig. 1). These additions were made in such a manner that the serum protein concentrations were unchanged. The hemoglobin was prepared by washing the red cells from the same blood sample six times with 10 volumes of physiological saline and laking them with distilled water. The concentration of sodium chloride was then brought up to 1.2 per cent and the stroma removed by centrifugation at 40,000 R.P.M. for 30 minutes in the ultracentrifuge. The optical densities of the serum both before and after the addition of hemoglobin were read in a Beckman spectrophotometer at wave-length 576 mμ. Before making these latter readings, all samples were diluted 1:10 with the buffer

in order to allow the readings to be made in the most sensitive range of the instrument. It is evident from the data in Table I that both the optical density and the area of γ-globulin increase by an amount proportional to the amount of hemoglobin added. It would therefore be possible to determine the amount of hemoglobin present in sera and its effect on the electrophoretic pattern from the optical density, provided one could estimate the optical density of the non-hemolyzed serum. The other substances in serum besides hemoglobin, such as the bile pigments and lipides which either absorb or scatter light, make attempts to cor-

TABLE I

Effect of Measured Quantities of Hemoglobin on Pattern Areas of Serum

Material	Nitrogen	Pattern area, planimeter units						Optical density*
		Hemo-globin	Albumin	Globulins				
				α_1	α_2	β	γ	
Phosphate buffer								
		mg. per cc.						
Clear serum	3.71		425		45	110	90	0.020
Hb	0.36	85						0.249
Serum + Hb	4.08	170	415		38	128	175	0.270
Hb	0.72	170						0.497
Serum + Hb	4.41		415		38	128	260	0.518
Barbiturate buffer								
Clear serum				405	39	58	97	68
Hb	0.36	82						
Serum + Hb				416	37	59	183	72

* Beckman spectrophotometer readings at λ = 576 mμ on solutions after dilution with 9 parts of buffer.

rect for hemolysis precarious unless the effect of these other substances is specifically measured.

In this laboratory, it has been found possible to discern non-hemolyzed serum by its appearance but impossible to estimate subjectively the quantity of hemoglobin in hemolyzed serum.

When these same amounts of hemoglobin were added to the same serum in barbiturate buffer, the area of β-globulin was similarly increased, as is illustrated in Fig. 2 and Table I.

Dialysis—Reiner and Fenichel (12) have correlated dialysis rate with stirring and temperature. In order to show the effect of dialysis on the electrophoretic pattern of serum, analyses were made on a serum sample without any dialyses and after dialysis of 24 and 48 hours. Parallel ex-

periments were made in two different buffers, *viz.*, the phosphate-saline and the barbiturate buffers described above.

Sample II—A sample of fresh serum was divided into two aliquots (each of these being later divided into three subaliquots). Each of the two aliquots was diluted with 2 parts of the respective buffers. A portion of each was immediately taken for electrophoresis. The remainder was put into bags made from viscose sausage casings (Visking) whose average pore diameter, measured from rate of water flow under constant pressure, was about 2.5 mμ. The dialysis was allowed to take place in the cold without stirring. The volume of the dialysate was about 300 times that of the original serum. After 24 hours, a sample was removed from the bag, the remainder being left to dialyze in the same bag against one-half
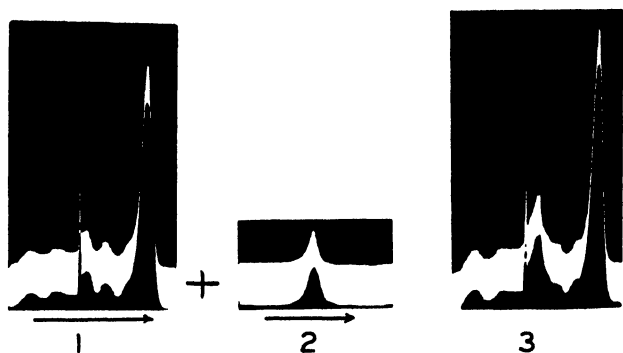
Fig. 2. Serum patterns showing the effect of hemolysis in barbiturate buffer. (*1*) non-hemolyzed serum; (*2*) 0.23 per cent hemoglobin; (*3*) serum plus hemoglobin.

of the same buffer for an additional 24 hours. The results of this experiment are recorded in Table II and the patterns are shown in Fig. 3. This experiment was repeated in different samples of sera four times with similar results.

In the phosphate buffer the apparent proportion of albumin increased slightly after dialysis for 1 day, then returned after 2 days to about the value obtained with no dialysis. The proportion of α- and β-globulins did not change, whereas γ-globulin showed a slight decrease after the 1st day but increased to a little above the undialyzed value after the 48 hours dialysis. None of these changes with time of dialysis, however, is of significance because all of the values fall nearly within the error of measurement (see below).

In the veronal buffer the patterns appear to be different but the measurement of each component does not reveal very significient changes except in the δ- and ϵ-boundaries and in the mobilities.

TABLE II

Correlation of Dialysis Time with Electrophoresis Patterns, Conductivities, and Relative Refractivities

Dialysis time	Descending or ascending	Pattern area, planimeter units							Mobilities, 10⁻⁶ cm.² per volt-sec.					Relative refractivities*		Conductivities, 10⁻³ mho	
		Albumin	α₁-Globulin	α₂-Globulin	β-Globulin	γ-Globulin	ε- or δ-Globulin	Total	Albumin	α₁-Globulin	α₂-Globulin	β-Globulin	γ-Globulin	Buffer vs. water	Protein vs. buffer	Buffer	Protein
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)
						Phosphate buffer											
hrs. 0	D.	447		48	141	119		755	4.7		3.2	2.1	0.3	1.01	2.43	9.8	8.74
	A.	415	30	44	117	143		749	4.6	4.1	3.1	2.3	0.6				
	D., %	59.2		6.3	18.7	15.8											
24	D.	423		43	122	99		687	4.7		3.2	2.2	0.5	1.01	2.14	9.8	9.28
	A.	375	25	28	110	125		663	4.8	4.3	3.5	2.5	0.4				
	D., %	61.5		6.2	17.8	14.4											
48	D.	424		48	133	120		725	4.5		3.0	2.0	0.3	1.01	2.17	9.8	9.31
	A.	395		38	112	145		690	4.6		3.4	2.3	0.4				
	D., %	58.5		6.6	18.3	16.6											
						Barbiturate buffer											
0	D.	447	69	84	135	77	0	821	5.7	4.8	3.7	2.5	0.8	2.42	1.9	3.11	4.35
	A.	460	60	67	149	69	21	826	5.6	4.6	3.7	2.5	0.9				
	D., %	55.2	8.5	10.3	16.6	9.4											
24	D.	447	62	58	129	74	52	730	6.2	5.3	4.1	2.9	1.1	2.42	2.26	3.11	3.04
	A.	355	50	58	127	79	97	766	6.5	5.5	4.5	3.1	1.3				
	D., %	55.2	9.1	8.6	19.0	10.9											
48	D.	447	50	52	117	68	50	685	5.5	4.6	3.7	2.5	0.9	2.42	2.31	3.11	3.08
	A.	355	43	45	100	51	71	665	5.8	5.0	4.0	2.7	1.0				
	D., %	55.0	7.8	8.1	18.4	10.7											

* Zeiss interferometer readings with 1 cm. cell.

In Columns 15 and 16, interferometer readings are recorded, which give relative refractive index differences between the protein solutions and the buffer solutions measured just before these solutions are placed in the electrophoresis cell. Interferometer readings for the two buffers against water are recorded in Column 15 of Table II. These latter data show that the refractive increment caused by veronal solutes is about 2.4 times that caused by the phosphate-saline solutes, although
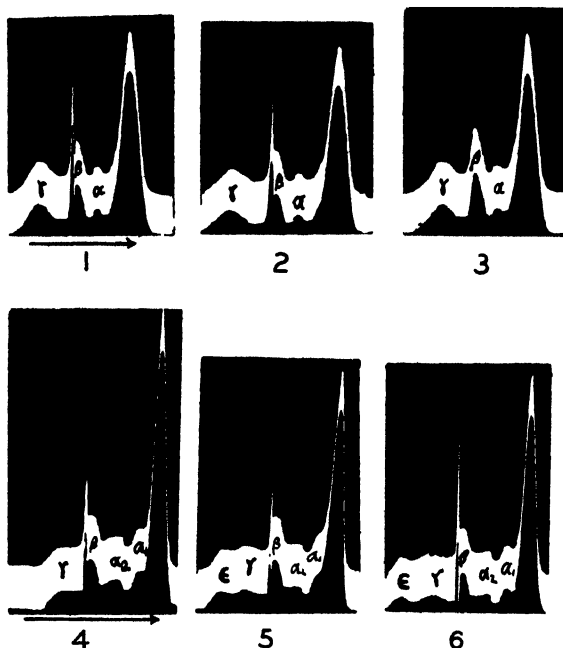
Fig. 3. Serum patterns showing the effect of dialysis. (*1*) and (*4*) undialyzed; (*2*) and (*5*) dialyzed 1 day; (*3*) and (*6*) dialyzed 2 days; (*1*), (*2*), and (*3*) in phosphate buffer; (*4*), (*5*), and (*6*) in barbiturate buffer.

the ionic strength of the veronal buffer is only one-half that of the phosphate. The refractive increments caused by the diluted serum read against the buffer (or dialysates) are recorded in Column 16. The conductivities of the buffer solutions (or dialysates) are recorded in Column 17 and those of the protein solution are recorded in Column 18 of Table II.

Mobility data were calculated by using buffer conductivities for the descending boundaries and protein solution conductivities for the ascending boundaries. This procedure gives better agreement between the two legs of the cell. When the samples were analyzed in barbiturate

buffer without dialysis, the movement of boundaries on the ascending side was considerably less than on the descending side, because the field strength here was reduced by the migration of chloride ions from the serum into this region ahead of the albumin.

Storage—It has been observed that when serum or plasma is allowed to stand a few days at room temperature or is shipped without refrigeration there occur rather marked changes in the α- and β-globulin portion of the electrophoretic pattern. In order to show these changes precisely, the following experiment was performed.

Sample III—Venous blood from a normal subject was allowed to clot in clean sterile tubes for 2 hours at room temperature. The blood was then centrifuged for ½ hour at about 1500 R.P.M., whereupon the clear, unhemolyzed serum was decanted. Three aliquots were then sealed in sterile ampules and treated in the following manner before electrophoretic
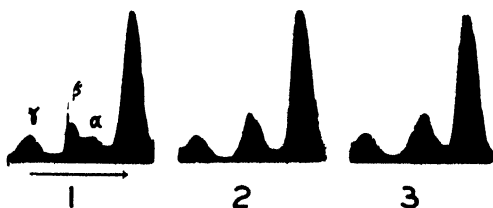
Fig. 4. Electrophoretic patterns of the same sample of serum in phosphate buffer. (*1*) stored in ice box 15 days; (*2*) shipped; (*3*) stored at room temperature 8 days.

analysis: Aliquot 1, placed in the ice box at 2–6° for 15 days; Aliquot 2, shipped from New York to Dallas and back by air mail (8 days); Aliquot 3, left at room temperature (20–25°) for 8 days. The difference in these patterns is shown in Fig. 4.

Sample IV—When plasma taken from whole blood (citrated) which had been stored in the cold (blood bank) for 3 weeks was compared with a portion of the same sample which was analyzed before storage, changes also occurred in the α- and β-globulin region, as portrayed in Fig. 5. Since it has been demonstrated (13, 14) that lipides are associated with these two globulins, a group of experiments was performed to study alterations in the pattern produced by various methods of lipide removal and aging.

Sample V—This sample of serum prepared in the same manner as Sample III was divided into ten aliquots and treated as follows: Aliquot 1, untreated; Aliquot 2, filtered in a Seitz filter; Aliquot 3, ether-extracted three times by the McFarlane method (13); Aliquot 4, a portion of Aliquot 3 stored at room temperature for 5 days; Aliquot 5, ultracentrifuged at 40,000 R.P.M. for 40 minutes, whereupon a fatty layer was discarded,

stored at room temperature for 5 days, and ultracentrifuged again to remove fats; Aliquot 6, stored for 12 days at room temperature; Aliquot 7, a portion of Aliquot 6 after ether extraction three times by the McFarlane method; Aliquot 8, heated at 57° for 10 minutes; Aliquot 9, a portion of Aliquot 8, ether-extracted; Aliquot 10, heated at 60° for 15 minutes. The
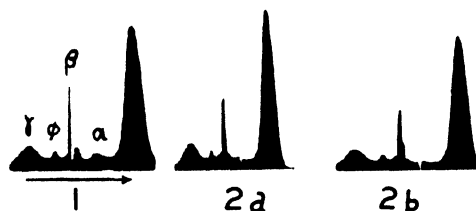
FIG. 5. Plasma patterns in phosphate buffer. (*1*) fresh; (*2*) after storage with cells in blood bank for 21 days; (*2a*) after electrophoresis for 135 minutes; (*2b*) 165 minutes, showing the mobility of the inverted refractive gradient at the α-globulin boundary.
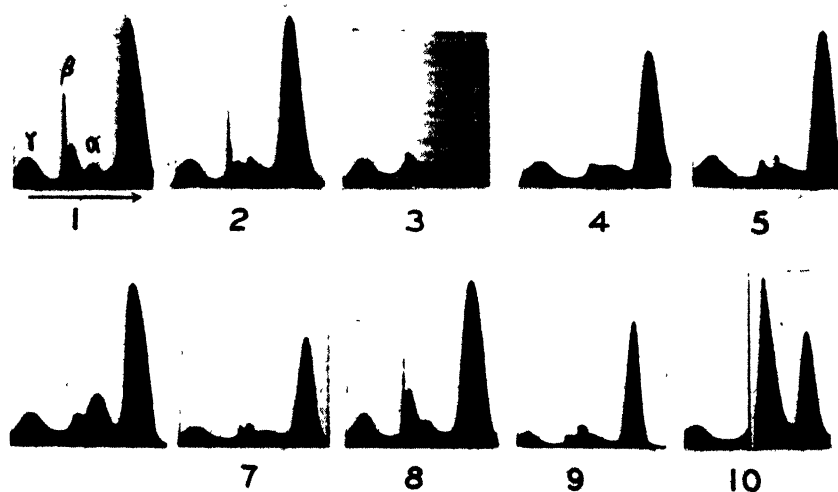
FIG. 6. See the text, Sample VI

results of these experiments are shown in Fig. 6. As was pointed out by McFarlane (13), low temperature freezing of the serum-ether emulsion removes only part of the total lipides, but removes little or no nitrogen from human serum.[1] Repeated extractions with ether do not appreciably affect the electrophoresis patterns. Faber[1] has shown that most of the cholesterol is removed at the first extraction, although small quantities

[1] Personal communication from M. Faber.

of phospholipides may continue to be removed even at the tenth or twelfth extraction.

In order to observe the effect of other treatments of serum the following experiment was undertaken.

Sample VI—A sample of fresh serum was divided into six aliquots and treated as follows: Aliquot 1, control; Aliquot 2, frozen in a dry ice-alcohol
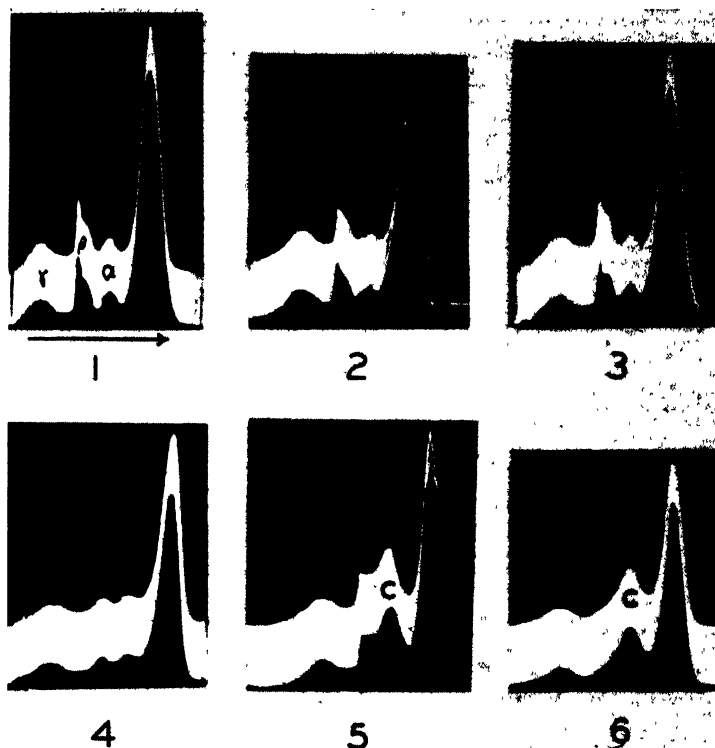
Fig. 7. Serum in phosphate buffer. (*1*) fresh; (*2*) frozen; (*3*) lyophilized; (*4*) ether-extracted; (*5*) heated; (*6*) heated and ether-extracted (see the text, Sample VII).

bath and allowed to thaw in the ice box; Aliquot 3, lyophilized, then restored to the original serum volume by adding distilled water; Aliquot 4, ether-extracted three times by the McFarlane method; Aliquot 5, heated at 58° for 20 minutes; Aliquot 6, a portion of Aliquot 5 after ether extraction three times by the McFarlane method. The results of these experiments are illustrated in Fig. 7 and the measurements on the patterns are collected in Table III. From these patterns it is apparent that both freezing and thawing and lyophilization cause changes in the electro-

phoretic pattern, so that it would be unfair to compare fresh sera with samples which had been stored by freezing or lyophilization.   In both treated samples there appeared considerable opalescence which migrated with the α-globulin (see Fig. 6, patterns 3 and 7; also Fig 7).   The areas representing albumin components were considerably reduced and γ-globulin became both larger and more diffuse.   This alteration probably represents some albumin degeneration, because enzymatic or other methods of hydrolysis tend to destroy the electrophoretic properties of proteins.   Heating at 58° for 20 minutes (pattern 5, Fig. 7) caused the formation of a component designated by van der Scheer, Wyckoff, and Clarke (2) as the C component, which was not diminished by ether extraction (pattern 6). The total amount of material removed by ether extraction here was independent of heating (compare patterns 4 and 6).   In another experiment

TABLE III

Effect of Various Manipulations on Electrophoretic Patterns of Serum Carried out in phosphate buffer.

Treatment	Pattern area, planimeter units			
	Albumin	Globulins		Total
Untreated	435	48	86	68	637
Frozen and thawed	390	50	85	78	603
Lyophilized	402	47	97	88	634
Ether-extracted	324	47	47	64	482
Heated 20 min. at 58°	352	143	63	74	632
" 20 " " 58° and extracted	295	143		50	488

not shown, serum was heated after extraction with ether, whereupon the C component appeared and was not affected by further similar extractions.

It would appear from the foregoing experiments that changes which occur readily at room temperature or by the usual methods of serum manipulation and storage are mainly concerned with the lipide content of the serum, whereas the changes produced by more drastic temperatures would seem to depend upon the denaturation of the proteins, a process independent of the presence of lipides.

Serum obtained from the same subject, after fasting 4 hours and 18 hours after a fatty meal, appeared markedly different in the test-tube but revealed no significant electrophoretic differences.

Measurement of Patterns—The data of Table IV show the deviations in measurements obtained when three different people trace and measure with a planimeter the same pattern.   The symbols in the first column stand for the person making the measurement.   These data indicate that

the variation in different measurements is just as great when made by the same individual as when made by different ones. These patterns were made by a modification (11) of the scanning method of Longsworth (10) in which the scanning diaphragm in front of the camera lens consisted of two edges forming a wide slit. Each datum in Table IV represents, therefore, the mean of the measurements made on the top and bottom patterns. This procedure eliminated most of the error resulting from the indeterminateness of the pattern edge and from variation in light exposure which here was small but in some cases may be appreciable (11). The greatest source of error is in establishing the base-line. In order to minimize this error, the initial boundaries were forced by compensation well out from behind the cell plates, so that after electrophoresis a portion of

TABLE IV

Repeated Measurements of Pattern Area

Person	Albumin	Globulins			Total
		α	β	γ	
M.	420	49	134	127	730
"	438	50	133	·120	741
S.	420	48	125	120	713
C.	425	45	137	115	722
"	418	49	134	117	718
Average................................	424	48	133	120	725

the gradient curve was horizontal in the region adjacent to the non-moving boundary. Similarly, the leading boundary was not allowed to traverse the cell completely, thus leaving a portion of horizontal curve there. These horizontal portions on each side of the pattern establish the ends of the base-line. If the optical parts in the apparatus are flawless and the light intensity is uniform across the pattern, the base-line may be drawn straight between the established ends.

In most instances, only the descending patterns were used for the area measurements because it has been shown (15–17) that the descending boundaries are less affected by superimposed gradients of either salt or protein. Both ascending and descending patterns were used for mobility measurements.

SUMMARY

In the electrophoretic analysis of hemolyzed biological substances, such as blood serum or plasma, the contribution of hemoglobin to the pattern may be estimated by subtracting the β-globulin area obtained in phos-

phate buffer from that obtained in barbiturate buffer, provided the samples are not dialyzed more than 24 hours.

The changes in the electrophoretic pattern caused by dialysis of serum in phosphate-saline buffer are insignificant. It appears feasible to analyze serum in this buffer without dialysis.

In veronal buffer at pH 8.6 the alterations in the pattern resulting from dialysis appear to be significant, but the actual measurements of the component areas reveal only small changes except for a general decrease in pattern area with dialysis time.

Evidence is given that serum should not be shipped except when refrigerated. It should be separated from the blood cells as soon as possible and maintained at ice box temperatures. Freezing or lyophilization produce measureable changes.

BIBLIOGRAPHY

1. Krejci, L. E., Sweeny, L., and Sanigar, E. B., *J. Biol. Chem.*, **158**, 693 (1945).
2. van der Scheer, J., Wyckoff, R. W. G., and Clarke, F. L., *J. Immunol.*, **40**, 39 (1941).
3. Hardt, C. R., Huddleson, I. F., and Ball, C. D., *J. Biol. Chem.*, **163**, 211 (1946).
4. Moore, D. H., and Mayer, M., *J. Biol. Chem.*, **156**, 777 (1944).
5. Tiselius, A., *Tr. Faraday Soc.*, **33**, 524 (1937).
6. Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, **67**, 119 (1939).
7. Longsworth, L. G., *Chem. Rev.*, **30**, 323 (1942).
8. Ross, V., Moore, D. H., and Miller, E. G., Jr., *J. Biol. Chem.*, **144**, 667 (1942).
9. Cohen, P. P., and Thompson, F. L., *J. Lab. and Clin. Med.*, **33**, 75 (1948).
10. Longsworth, L. G., *J. Am. Chem. Soc.*, **61**, 529 (1939).
11. Moore, D. H., and White, J. U., *Rev. Scient. Instruments*, **19**, 70 (1948).
12. Reiner, M., and Fenichel, R. L., *Science*, **108**, 164 (1948).
13. McFarlane, A. S., *Nature*, **149**, 439 (1942).
14. Moore, D. H., Martin Du Pan, R., and Buxton, C. L., *Am. J. Obst. and Gynec.*, **57**, 312 (1949).
15. Longsworth, L. G., and MacInnes, D. A., *J. Am. Chem. Soc.*, **62**, 705 (1940).
16. Svensson, H., *Ark. Kemi, Mineral. o. Geol.*, **17**, No. 14 (1943).
17. Svensson, H., *Ark. Kemi, Mineral. o. Geol.*, **22**, No. 10 (1946).

STUDIES ON THE CONVERSION OF RADIOACTIVE LEUCINE TO ACETOACETATE*

By MINOR J. COON and SAMUEL GURIN

(From the Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia)

Although it is well established that leucine gives rise to ketone bodies in the course of its metabolism in the mammalian organism, the mechanism of this transformation has not been fully elucidated. As early as 1906, Embden and his colleagues (2) presented evidence from perfusion studies that isovaleric acid is a ketogenic substance, and they proposed it as a possible intermediate in the biological degradation of leucine. Subsequently, Ringer, Frankel, and Jonas (3) reported that the administration of isovaleric acid to phlorhizinized dogs results in the excretion of extra ketone bodies. The latter investigators suggested that the compound undergoes reductive demethylation to form butyric acid, which is then oxidized directly to acetoacetic acid.

More recently, Bloch (4) has found that deuterium-labeled leucine or isovaleric acid is partially converted in the intact animal to "acetate," which can be utilized for acetylations as well as for cholesterol formation. These experiments are of particular interest in that they permit a calcula-tion of the extent to which acetate was formed from the administered compounds. Bloch's data indicate that only half a mole of acetoacetate may be formed from a mole of leucine.

The experiments described below were undertaken in the hope that the mechanism of these reactions could be established with the aid of the C^{14} isotope as a tracer. For this purpose, two preparations of leucine were synthesized, with the tag in the β and the γ positions, respectively. From a study of the metabolism of the two compounds in liver slices, it was possible to demonstrate that the α- and β-carbons of leucine furnish a 2-carbon fragment capable of condensing to form acetoacetate. The isopropyl group of the amino acid does not yield a similar 2-carbon compound to an appreciable extent.

EXPERIMENTAL

Synthesis of β-C^{14}-Leucine—The reactions which were employed are as follows:

$$(CH_3)_2CHMgBr + \overset{*}{C}O_2 \rightarrow (CH_3)_2CH\overset{*}{C}OOH \rightarrow (CH_3)_2CH\overset{*}{C}H_2OH \rightarrow$$

$$(CH_3)_2CH\overset{*}{C}H_2Br \rightarrow (CH_3)_2CH\overset{*}{C}H_2CHNH_2COOH$$
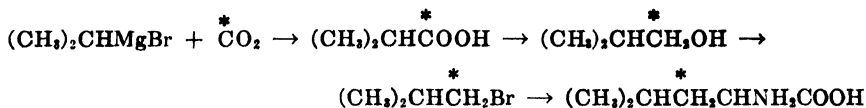
Carboxyl-labeled isobutyric acid was prepared by the Grignard reaction according to the general method of Sakami, Evans, and Gurin (5). The carbon dioxide formed by the action of perchloric acid on 10 gm. of radioactive barium carbonate was allowed to react with the Grignard reagent made from 6.20 gm. of isopropyl bromide. The yield of redistilled isobutyric acid was 59 per cent of the theoretical amount. The acid was converted directly to isobutyl alcohol by the use of lithium aluminum hydride, according to the general method of Nystrom and Brown (6). Treatment of the dry alcohol with phosphorus tribromide gave isobutyl bromide in a 52 per cent over-all yield from the acid. The isobutyl bromide was then condensed with acetylaminomalonic ester, according to the directions of Snyder, Shekleton, and Lewis (7), and the crude reaction product was hydrolyzed with hydrobromic acid. The free amino acid, which was obtained by the addition of ammonium hydroxide, was recrystallized from water. The yield from the isobutyl bromide was calculated as 42 per cent. After an additional recrystallization, the leucine gave a satisfactory value for total nitrogen content (micro-Kjeldahl), and it had a radioactivity of 603 counts per minute per mg. of carbon, or 3618 calculated for the β-carbon alone.

Synthesis of γ-C^{14}-Leucine—The reactions are as follows:

$$CH_3MgI + \overset{*}{C}O_2 \rightarrow CH_3\overset{*}{C}OOH \rightarrow (CH_3)_2\overset{*}{C}O \rightarrow (CH_3)_2\overset{*}{C}HOH \rightarrow$$

$$(CH_3)_2\overset{*}{C}HBr \rightarrow (CH_3)_2\overset{*}{C}HCOOH \rightarrow (CH_3)_2\overset{*}{C}HCH_2OH \rightarrow$$

$$(CH_3)_2\overset{*}{C}HCH_2Br \rightarrow (CH_3)_2\overset{*}{C}HCH_2CHNH_2COOH$$
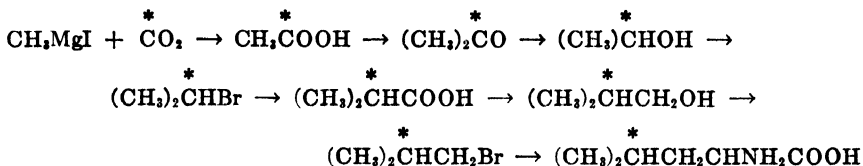
3 gm. of carboxyl-labeled acetic acid were prepared from radioactive barium carbonate and non-labeled methyl iodide (5). The acid was converted to the calcium salt and dried in an oven at 110°. It was then converted to acetone by heating at 430–490° in a glass tube approximately 15 cm. in length and 2 cm. in diameter. Dry, heated nitrogen gas was continuously passed through the system in order to carry the acetone vapors into a receiver cooled with liquid air. The product was washed into a reduction vessel with purified dioxane and shaken for 5 hours with Raney's nickel under hydrogen at a pressure of 40 pounds. The resulting isopropyl alcohol was separated by distillation and then treated with phosphorus tribromide to yield radioactive isopropyl bromide in an overall yield of 50 per cent from the acetic acid. The bromide was con-

verted to α-C¹⁴-isobutyric acid by carboxylation with non-radioactive carbon dioxide, and the acid was then transformed into γ-C¹⁴-leucine by the reactions previously described. The radioactivity of the analytically pure amino acid was 40 counts per minute per mg. of carbon, or 240 calculated for the γ-carbon alone.

Determination of Radioactivity—All of the radioactivity measurements reported in this paper were performed on barium carbonate samples. The various organic compounds were submitted to the wet oxidation procedure of Van Slyke (8), and the carbon dioxide which was produced was trapped in a saturated solution of barium hydroxide. The barium carbonate was thoroughly washed with water and then dried before it was spread on plates in a manner previously reported (9). The radioactivity of the samples was determined with a bell-shaped Geiger counter having a thin mica window (2.5 mg. per sq. cm.). Under these conditions, the relationship of recorded activity in the counter to plate thickness was found to be identical with that described by Reid (10).

The counts in excess of background were in all cases corrected to activity at infinite thickness. With the exception of a few values (0.6 or less) which are of questionable significance, the radioactivities reported are subject to a standard error in counting of no greater than 5 per cent.

Incubation of β-C¹⁴-Leucine with Liver Slices—The procedures described by Edson (11) and Cohen (12) for the study of leucine metabolism in liver tissue *in vitro* were adapted to a larger scale so as to provide sufficient material for a complete chemical degradation of the resulting acetoacetate. Adult rats which had been fasted for 24 hours were sacrificed. The livers were removed immediately, and tissue slices approximately 0.5 mm. in thickness were prepared. To 2.5 gm. of moist slices in a Warburg flask, 20 ml. of Ringer-phosphate buffer solution containing 3.0 mg. of the radioactive DL-leucine were added, and the flask was shaken for 4 hours at 38° under an atmosphere of oxygen. Respiratory carbon dioxide was absorbed by a 20 per cent solution of sodium hydroxide in the center well.

Upon the completion of the incubation, the medium was decanted and the slices were washed. The combined supernatant solutions and washings from four flasks were treated with the copper-lime reagents commonly used for deproteinization. To the filtrate, containing approximately 8 mg. of acetoacetic acid produced by the tissue, 90 mg. of non-radioactive acetoacetic acid freshly prepared from the ethyl ester (13) were added.

Degradation Procedure A—A portion of the final solution containing carrier acetoacetate was heated under a reflux with Denigès' reagent, and the carbon dioxide which was liberated was collected as barium carbonate.

The mercury-acetone complex was separated from the solution, dissolved in 1 N hydrochloric acid, and purified by steam distillation of the acetone into fresh mercuric sulfate reagent. It is apparent from the results given in Table I that in each experiment the radioactivity was concentrated in the acetone fraction.

Degradation Procedure B—In order to establish the radioactivity in the individual methyl and methylene carbons of the acetoacetate, use was made of the degradation procedure recently reported by Weinhouse and Millington (14). A portion of the protein-free filtrate containing carrier acetoacetate was oxidized with permanganate at 0° to yield carbonic,

TABLE I

Distribution of Radioactivity in Acetoacetate Formed from β-Labeled Leucine in Liver Slices

Compound	Carbons of acetoacetic acid represented	Counts per minute per mg. of carbon	
		Experiment 1	Experiment 2
Respiratory CO_2		7.4*	9.4*
Degradation Procedure A			
CO_2	COOH	0.6	0.6
Acetone	CH_3, CO, CH_2	9.3	10.3
Degradation Procedure B			
Formic acid	CH_2	12.2	15.4
$BaCO_3$	CO	0.6	0.5
Acetone	CH_3, CO, CH_3	7.7	8.4
	CH_3 (calculated)	11.6	12.6

* These figures cannot be compared directly with the other values in the table because the acetoacetate was diluted by carrier (see the text).

formic, and acetic acids from the carboxyl, α-, and β- + γ-carbons, respectively, of the acetoacetic acid. To distinguish between the β- and the γ-carbons, it was necessary to degrade the acetic acid fraction further. Accordingly, the acetic acid was converted to the barium salt, which was pyrolyzed in an evacuated sealed tube to form acetone and barium carbonate. The acetone was collected in a portion of the tube which was chilled in a chloroform-dry ice mixture; it was subsequently isolated as the mercury complex and then purified in the usual manner. The crude barium carbonate, which was gray in color, was acidified, and the liberated carbon dioxide was aerated into barium hydroxide solution.

The radioactivity of the formic acid samples, as shown in Table I, indicates the presence of relatively large amounts of C^{14} in the methylene carbon of the acetoacetic acid. In contrast, the $BaCO_3$ formed by pyrolysis of the barium acetate bears only a trace of the C^{14} isotope in each experi-

ment. The activity of the methyl carbon was calculated from that of the acetone produced by the pyrolysis of barium acetate, by multiplying by $\frac{2}{3}$.

It is apparent that in each experiment the radioactivity was concentrated almost entirely in the methyl and methylene carbons, and to approximately the same extent in each of these. It is therefore concluded that at some stage in the metabolism of leucine its α- and β-carbons split off as a 2-carbon intermediate which is capable of condensing to form acetoacetate. The two degradative procedures which were used yielded data which are in good agreement. In Experiment 1, the radioactivity of the individual methylene and methyl carbons of the acetoacetic acid, as determined in Procedure B, accounts for 85 per cent of the activity of the acetone fraction obtained in Procedure A. In Experiment 2, a similar calculation yields a value of 91 per cent.

Administration of β-C^{14}-Leucine to Phlorhizinized Rat—A fasted, phlorhizinized rat was injected subcutaneously with 100 mg. of β-C^{14}-leucine, and the acetoacetate which appeared in the urine during the following 24 hours was degraded by standard procedures. The mercury-acetone complex was found to be radioactive, and iodoform prepared from it accounted for almost all of the isotope present. These findings are in accord with the results of the tissue slice experiments.

Unpublished data indicate that in the intact phlorhizinized rat singly labeled radioactive acetoacetate is randomized to a considerable extent prior to its appearance in the urine. Therefore, in the present experiment no attempt was made to establish the distribution of C^{14} between the methyl and methylene carbons.

Incubation of γ-C^{14}-Leucine with Liver Slices—The results of these experiments, which were conducted in a manner similar to that already described for the β-labeled amino acid, are presented in Table II. In Experiment 1 no carrier acetoacetate was added to the protein-free filtrate, which was treated according to Procedure A. The appearance of isotope solely in the acetone fraction suggested the possibility that in the presence of the liver slices the isopropyl group of the amino acid had been directly converted to acetone. As a control experiment, a solution containing 6 mg. of γ-C^{14}-leucine and 8 mg. of non-radioactive acetoacetic acid was carried through the copper-lime treatment and the mercuric sulfate degradation. Under these conditions, the mercury-acetone complex had no measurable radioactivity. Therefore, the findings in Experiment 1 must be attributed to the action of the liver tissue on the amino acid.

In the event that the isopropyl group is converted to acetone during metabolism and that the latter compound is not further metabolized in

liver tissue, one would expect to find no radioactivity in any of the volatile acids produced by the action of permanganate. Acetone, unlike acetoacetate, is not degraded under the experimental conditions of this procedure. In Experiment 2, 8 mg. of acetoacetate carrier were added to the solution before aliquots were submitted to the mercuric sulfate and the permanganate degradations. The acetone fraction exhibited significant radioactivity, whereas the carbon dioxide liberated by Denigès' reagent did not. Of the volatile acids produced in Procedure B, only the acetate exhibited significant radioactivity. This finding was confirmed in Experiment 3, where 16 mg. of carrier acetoacetate were added to the

TABLE II

Distribution of Radioactivity in Acetoacetate Formed from γ-Labeled Leucine in Liver Slices

Compound	Carbons of acetoacetic acid represented	Counts per minute per mg. of carbon		
		Experiment 1	Experiment 2	Experiment 3
Respiratory CO_2		0.2	0	0.3
Degradation Procedure A				
CO_2	COOH	0	0	
Acetone	CH_3, CO, CH_2	5.1	2.9	
Degradation Procedure B				
CO_2	COOH		0	0.3
Formic acid	CH_2		0	0
Acetic "	CH_3, CO		3.1	4.1
$BaCO_3$	CO			8.2
Acetone	CH_3, CO, CH_3			2.8

combined copper-lime filtrate from eight flasks in order to provide sufficient material for degradation of the acetate fraction. The acetic acid was converted to the barium salt, which was pyrolyzed, and the products were purified. It is apparent that the radioactivity of the barium carbonate was exactly twice that of the acetate, which was therefore labeled only in its carboxyl carbon. The significance of these findings is discussed below.

DISCUSSION

In the course of their perfusion studies, Embden and his associates (2, 15) discovered that a greater amount of ketone bodies was produced from D- or DL-leucine than from the L isomer. Edson (11) confirmed this difference in behavior by his studies on the formation of acetoacetate from the L- or DL-amino acid in liver slices. Since the optical isomers

of the amino acid are known to yield the same keto acid upon oxidative deamination (16, 17), one would not expect their carbon chains to meet with different catabolic fates. It would appear that the rate of deamination is the factor governing the differences observed by these workers. Rose (18) has established the fact that D-leucine is not an efficient substitute for the L isomer in stimulating growth in the rat. The rate of the inversion is apparently inadequate to meet the demands of the organism for this purpose. That the inversion actually can occur to a certain extent was proved by Ratner, Schoenheimer, and Rittenberg (19). They concluded from isotopic experiments that complete deamination of the D isomer must take place prior to asymmetrical amination. Accordingly, the conclusions reached in the present investigation with respect to the biological degradation of the carbon chain are believed to apply to both enantiomorphs of leucine.

The data from the studies with the β-labeled leucine lead to the conclusion that during the metabolism of the amino acid its α- and β-carbons split off as a 2-carbon intermediate which can condense to form acetoacetate. The experimental evidence definitely rules out the hypothesis that 4 carbons of the amino acid might form acetoacetate directly, for in such an event it would have been labeled in only a single position. This deduction depends upon the well established fact that acetoacetate does not undergo randomization in liver tissue (20).

Our findings are in agreement with the possibility that isovaleric acid is an intermediate in the reactions under discussion. However, butyric acid could not have been formed from isovaleric acid, as was proposed at one time by Ringer et al. (3). Under the experimental conditions of the present study, butyrate would have formed acetoacetate with a greater proportion of the isotope in the methylene carbon (21). In the two experiments given in Table I, the ratios of the radioactivity of the methylene carbon to that of the methyl carbon are only 1.1 and 1.2, respectively.

In the *in vitro* experiments with the β-C^{14}-leucine, the respiratory carbon dioxide had appreciable radioactivity. These figures cannot be directly compared with the other data in Table I because the acetoacetate underwent chemical dilution with the carrier before it was degraded. Whereas roughly 10 per cent of the administered counts appeared in the acetoacetic acid, less than 1 per cent was in the respiratory carbon dioxide. It would seem likely that the latter effect is not evidence for an additional pathway of leucine metabolism, but an indication that a small portion of the 2-carbon fragments was oxidized via the tricarboxylic acid cycle. The values for the radioactivity of the carboxyl and carbonyl carbons of the acetoacetate are so low that they are of questionable significance.

The absence of significant amounts of radioactivity from the respira-

tory carbon dioxide in each experiment in Table II indicates that the isopropyl group of leucine is not completely oxidized in liver tissue. It can be concluded from the studies with the γ-labeled leucine that the isopropyl group does not furnish a 2-carbon fragment capable of giving rise to doubly labeled acetoacetate. The data support the conclusions previously reached with β-C^{14}-leucine as to the possible rôle of isovaleric acid in these reactions.

It remains to be established whether the isopropyl group is converted to a single compound which both forms an insoluble complex with Denigès' reagent and is readily oxidized to acetate by the action of permanganate. The true intermediate most likely is not a glycogenic substance, because no reliable evidence exists for the metabolic conversion of leucine to extra carbohydrate.

The accompanying series of reactions, showing the proposed metabolic pathway of leucine catabolism, is in accord with the evidence which has been presented.

$$(CH_3)_2\overset{\dagger}{C}H\overset{*}{C}H_2CHNH_2COOH \longrightarrow (CH_3)_2\overset{\dagger}{C}H\overset{*}{C}H_2COCOOH \longrightarrow$$

$$(CH_3)_2\overset{\dagger}{C}H\overset{*}{C}H_2COOH \longrightarrow [(CH_3)_2\overset{\dagger}{C}H{-}] + [{-}\overset{*}{C}H_2COOH]$$

$$\Big\downarrow$$

$$(?) \qquad \overset{*}{C}H_3CO\overset{*}{C}H_2COOH$$
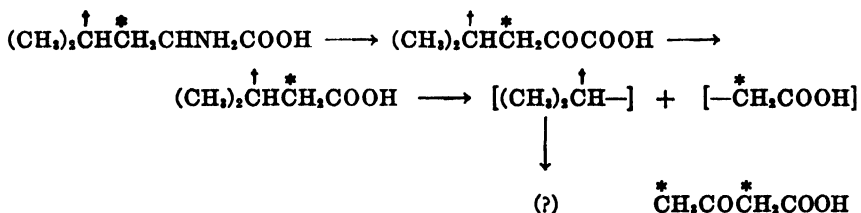
We wish to express our appreciation to Dr. Sidney Weinhouse for making available to us the unpublished details of his procedure for the degradation of acetoacetate with permanganate, and to thank Dr. D. Wright Wilson for his advice and criticism.

SUMMARY

It has been demonstrated by the incubation of β-C^{14}-leucine with liver slices that the α- and β-carbons of the amino acid split off as a 2-carbon intermediate which is capable of condensing to form acetoacetate. The findings are consistent with the hypothesis that isovaleric acid is an intermediate in leucine catabolism, but not with the assumption that isovalerate undergoes reductive demethylation to form butyrate or oxidative demethylation to form acetoacetate directly.

The administration of the β-labeled amino acid to a phlorhizinized rat, followed by degradation of the urinary acetoacetate, gave results in accord with the findings *in vitro*.

Evidence has been presented that the isopropyl group of leucine is not

completely oxidized to carbon dioxide in liver tissue, and that it does not furnish to an appreciable extent a 2-carbon fragment similar to the one arising from the α- and β-carbons of the amino acid.

BIBLIOGRAPHY

1. Coon, M. J., Gurin, S., and Wilson, D. W., *Federation Proc.*, **8**, 192 (1949).
2. Embden, G., Salomon, H., and Schmidt, F., *Beitr. chem. Physiol. u. Path.*, **8**, 129 (1906).
3. Ringer, A. I., Frankel, E. M., and Jonas, L., *J. Biol. Chem.*, **14**, 525 (1913).
4. Bloch, K., *J. Biol. Chem.*, **155**, 255 (1944).
5. Sakami, W., Evans, W. E., and Gurin, S., *J. Am. Chem. Soc.*, **69**, 1110 (1947).
6. Nystrom, R. F., and Brown, W. G., *J. Am. Chem. Soc.*, **69**, 2548 (1947).
7. Snyder, H. R., Shekleton, J. F., and Lewis, C. D., *J. Am. Chem. Soc.*, **67**, 310 (1945).
8. Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, **136**, 509 (1940).
9. Gurin, S., Delluva, A. M., and Wilson, D. W., *J. Biol. Chem.*, **171**, 101 (1947).
10. Reid, A. F., Preparation and measurement of isotopic tracers, Ann Arbor, 83 (1946).
11. Edson, N. L., *Biochem. J.*, **29**, 2498 (1935).
12. Cohen, P. P., *J. Biol. Chem.*, **119**, 333 (1937).
13. Ljunggren, G., *Biochem. Z.*, **145**, 422 (1924).
14. Weinhouse, S., and Millington, R. H., *J. Biol. Chem.*, **175**, 995 (1948).
15. Embden, G., *Beitr. chem. Physiol. u. Path.*, **11**, 348 (1908).
16. Krebs, H. A., *Biochem. J.*, **29**, 1620 (1935).
17. Blanchard, M., Green, D. E., Nocito, V., and Ratner, S., *J. Biol. Chem.*, **155**, 421 (1944).
18. Rose, W. C., *Physiol. Rev.*, **18**, 109 (1938).
19. Ratner, S., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **134**, 653 (1940).
20. Buchanan, J. M., Sakami, W., and Gurin, S., *J. Biol. Chem.*, **169**, 411 (1947).
21. Medes, G., Weinhouse, S., and Floyd, N. F., *J. Biol. Chem.*, **157**, 35 (1945).

FATTY ACID SYNTHESIS BY ENZYME PREPARATIONS OF CLOSTRIDIUM KLUYVERI*

IV. THE PHOSPHOROCLASTIC DECOMPOSITION OF ACETOACETATE TO ACETYL PHOSPHATE AND ACETATE

By E. R. STADTMAN and H. A. BARKER

(From the Division of Plant Nutrition, University of California, Berkeley)

Cell-free enzyme preparations of *Clostridium kluyveri* have been shown to oxidize ethanol and acetaldehyde to acetyl phosphate (17) and to convert a mixture of acetyl phosphate and acetate to butyrate with hydrogen as a reductant (18). Almost nothing is known about the mechanism of the conversion of acetyl phosphate and acetate to butyrate, but it has been postulated by Lipmann (15) that the first step is a condensation to form acetoacetate, which is then reduced (equation (1)).

$$CH_3COOPO_3^- = + CH_3COO^- \xrightarrow{- HPO_4^-} CH_3COCH_2COO^- \xrightarrow{+2H_2}$$

$$CH_3CH_2CH_2COO^- + H_2O \quad (1)$$
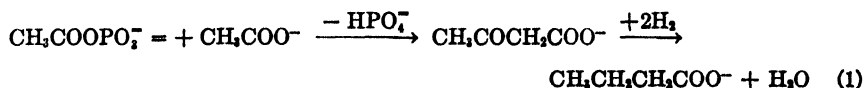
Although there is no direct evidence that acetoacetate is a precursor of butyrate, it is known that acetoacetate can be formed from acetate by both animal tissues (11, 19, 23) and butyric acid bacteria (7). It therefore seemed desirable to investigate the rôle of acetoacetate in butyric acid synthesis by the soluble enzymes derived from *C. kluyveri*. The experimental results demonstrate that acetoacetate can be decomposed in at least two ways, but they also prove that acetoacetate is not an intermediate in the formation or oxidation of butyrate.

Methods

Acetoacetate was determined manometrically by measuring the CO_2 evolved when this substance is catalytically decomposed with aniline acetate, according to the method of Jowett and Quastel (11).

·The acetoacetate used in this investigation was prepared by alkaline hydrolysis of ethyl acetoacetate (Eastman Kodak Company) by the method of Davies (8). Aqueous stock solutions of the sodium salt were kept at $-18°$.

The increase in inorganic phosphate produced by 7 minutes hydrolysis at 100° in 1 N HCl was taken as a measure of adenyl pyrophosphate. The adenosine-5-phosphoric acid was obtained from the Sigma Chemical Company.

β-Hydroxybutyrate was determined by the method of Behre and Benedict (2, 3).

The doubly labeled acetate ($C^{14}H_3C^{14}OOH$) was prepared by the fermentation of glucose in the presence of C^{14}-labeled bicarbonate by the anaerobic bacterium *Clostridium thermoaceticum* (5).

Butyric acid, labeled in the carboxyl group with C^{14}, was prepared by the Grignard method (5).

Determination of C^{14} in Acetoacetate—The acetoacetate was decomposed to carbon dioxide and acetone with aniline acetate (11). The carbon dioxide derived from the carboxyl group of the acetoacetate was precipitated as barium carbonate and radioactivity measurements were made on the precipitate. The acetone derived from the α-, β-, and γ-carbon atoms of the acetoacetate was converted to the insoluble 2,4-dinitrophenylhydrazone and radioactivity measurements were made directly on this derivative.

The decarboxylation of acetoacetate with aniline acetate was carried out in a diffusion apparatus (5, 21). In one arm of the apparatus was placed a suitable aliquot (1 to 2 ml.) of the sample to be tested. The sample was acidified (pH 4.0, brom cresol green indicator) with 1 N H_2SO_4, and 0.8 ml. of 0.1 M acetate buffer (pH 4.0) and 1 to 2 drops of tributyl citrate were added to prevent foaming. The system was shaken *in vacuo* several minutes to remove dissolved carbon dioxide. Finally a small vial (7 × 13 mm.) containing 0.4 ml. of aniline hydrochloride solution (120 mg.) was placed in the arm containing the sample, care being taken not to mix the aniline hydrochloride with the sample. In the other arm of the diffusion apparatus was placed 1 to 2 ml. of 0.3 N $Ba(OH)_2$. The apparatus was then assembled and evacuated. After evacuation, the aniline hydrochloride was tipped into the sample and the mixture was warmed to about 40° for 40 minutes and then all of the liquid in the sample was distilled *in vacuo* into the barium hydroxide. The excess barium hydroxide was titrated to the phenol red end-point. The barium carbonate was separated by centrifugation, washed twice with 1 ml. portions of CO_2-free water, and finally with 10 ml. of ethanol. The precipitate was spread uniformly on an aluminum disk, dried at 100°, and radioactivity measurements were made with a Geiger-Müller counter (5).

When the above procedure is followed, the acetone produced in the decomposition of acetoacetate is contained in the supernatant from the $BaCO_3$ precipitation. To separate the acetone from small amounts of

acetate that are also present, the supernatant was adjusted to pH 8.5 and distilled. To the distillate were added 10 ml. of saturated 2,4-dinitrophenylhydrazine reagent in 2 N HCl. The acetone hydrazone was separated by centrifugation, washed twice with 1 ml. portions of water, recrystallized from warm alcoholic solution, and finally dissolved in ethanol and transferred to aluminum disks, dried, and used for radioactivity measurements.

All other methods used in this investigation have been described elsewhere (16–18).
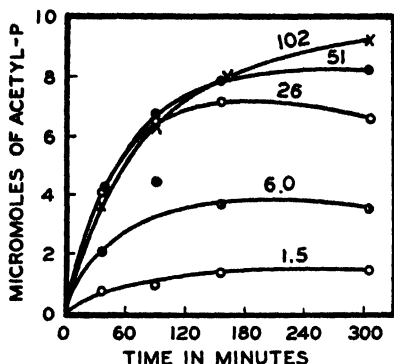
FIG. 1. The effect of inorganic phosphate on acetyl phosphate formation from acetoacetate. Each Thunberg tube contained 69 μM of acetoacetate, 20 mg. of dry cell-free extract (Lot D), and varying amounts of inorganic phosphate. The total volume was 2.0 ml. The numbers on the various curves refer to the micromoles of inorganic phosphate added per ml. The tubes were evacuated and stored at 26°. The pH was 5.7. The ordinate refers to micromoles of acetyl phosphate per ml.

Results

Phosphoroclastic Decomposition of Acetoacetate—When acetoacetate is incubated with cell-free extracts of *C. kluyveri* in phosphate buffer, it is rapidly decomposed and acetyl phosphate is formed. The dependence of acetyl phosphate formation on the presence of inorganic phosphate is evident from the data presented in Fig. 1, which show, as is to be expected, that, in the absence of inorganic phosphate, little if any acetyl phosphate is formed. Increasing the concentration of inorganic phosphate over a range of 1.5 to 102 μM per ml. caused a progressive increase in the total amount of acetyl phosphate produced. However, there does not appear to be any simple relation between the inorganic phosphate level and acetyl phosphate formation. Except at the highest phosphate level, the formation of acetyl phosphate was complete after 2 hours. The addition of more inorganic phosphate, or acetoacetate, at the time when acetyl phos-

phate formation ceased did not cause a resumption of the reaction. This indicates that the enzyme system was inactivated.

$$CH_3COCH_2COO^- + HPO_4^- \rightarrow CH_3COOPO_3^- + CH_3COO^- \qquad (2)$$

If acetyl phosphate is produced by a phosphoroclastic reaction of the type postulated by Lipmann (15) (equation (2)), then for each mole of acetoacetate decomposed 1 mole each of acetate and acetyl phosphate

TABLE I

Phosphoroclastic Splitting of Acetoacetate

Aceto-acetate, initial	Pi, initial	Δ aceto-acetate*	Δ acetyl P†	Δ acetyl P + acetate‡	Δ acetate§	Δ Pi‖	Δ acetyl P + acetate / Δ acetoacetate	Δ acetyl P / Δ Pi
				Experiment 1				
μM	μM	μM	μM	μM	μM	μM		
69	1.5	−4.0	1.4	9.0	7.6		2.25	
69	6.0	−7.5	3.5	14.7	11.2		1.96	
69	26	−11.0	6.6	23.2	16.6		2.10	
69	51	−12.5	8.2	26.0	17.8		2.08	
69	102	−16.5	9.2	28.0	18.6		1.70	
				Experiment 2				
175	105	−61.5	32.5	115	82.5	−30.5	1.87	1.06
337	105	−72.5	40.5	150	109.5	−38.0	2.07	1.06

Experiment 1, see Fig. 1 for experimental details. Experiment 2, 85 mg. of dry cell-free enzyme preparation in 5.0 ml. of 0.021 M phosphate buffer, pH 5.7, and the indicated amounts of acetoacetate incubated *in vacuo* in Thunberg tubes for 4 hours at 26°.

* Aniline acetate method.
† Hydroxamic acid method.
‡ Estimated by microtitration of the steam-volatile acids.
§ Obtained by difference, two preceding columns.
‖ Estimated by calcium precipitation method.

should be formed. To determine whether this was the case, samples from the experiments described in Fig. 1 were analyzed to see whether corresponding changes in acetoacetate and acetate concentrations had taken place. The results are summarized in Table I (Experiment 1). Also reported in Table I are the results of a similar experiment (No. 2) in which the uptake of inorganic phosphate was measured. The data show that for each mole of acetoacetate decomposed 2 moles of acetate (free acetate + acetyl phosphate) were formed. The disappearance of inorganic phosphate was equivalent to the amount of acyl phosphate formed (Experiment 2). A comparison of the amounts of acetate and acetyl phos-

phate produced shows that at least twice as much acetate was produced as acetyl phosphate. This is at variance with equation (2) according to which equal amounts of acetate and acetyl phosphate should be formed. Two possible explanations for this result can be suggested. (a) The acetoacetate may be decomposed by two different pathways; namely, by a hydrolytic cleavage to 2 molecules of acetate, and by a phosphoroclastic splitting, as described by equation (2). If both processes occurred simultaneously, a relatively greater accumulation of acetate than acetyl phosphate would occur. (b) The acetoacetate may be decomposed only by reaction (2), but part of the acetyl phosphate formed may be hydrolyzed to acetate. The latter explanation seems more reasonable in view of the fact that the rate of acetyl phosphate hydrolysis observed in other studies (to be reported) was sufficiently high to account for the disparity between the acetate and acetyl phosphate found in the present experiment.

Decomposition of Acetoacetate in Presence of Adenylic Acid—In experiments to be described later, it was found that some enzyme preparations were able to catalyze a rapid transfer of the phosphate group of acetyl phosphate to adenylic acid, with the formation of adenyl pyrophosphates. With this finding, the opportunity presented itself of determining experimentally whether or not acetoacetate was decomposed only by way of reaction (2). This point could be settled by studying the decomposition of acetoacetate in the presence of an excess of adenylic acid. Under these conditions, as rapidly as acetyl phosphate is formed, it enters into a reaction with adenylic acid to give acetate and adenyl pyrophosphate. The amount of pyrophosphate thus formed gives a measure of the acetyl phosphate produced. In this manner, the loss of high energy phosphate by hydrolysis of acetyl phosphate can be avoided.[1] Lipmann (15) made use of this same principle to obtain quantitative data on the formation of acetyl phosphate in the oxidative decarboxylation of pyruvate by enzyme preparations of *Lactobacillus delbrueckii*.

Acetoacetate decomposition in the presence of adenylic acid can be described by equations (3) and (4).[2]

$$CH_3COCH_2COO^- + HPO_4^- \rightarrow CH_3COOPO_3^- + CH_3COO^- \quad (3)$$
$$CH_3COOPO_3^- + AMP \rightarrow CH_3COO^- + ADP \quad (4)$$

The net reaction would be

$$CH_3COCH_2COO^- + HPO_4^- + AMP \rightarrow 2CH_3COO^- + ADP \quad (5)$$

from which it follows that for each mole of acetoacetate decomposed, 2 moles of acetic acid and 1 mole of pyrophosphate should be formed. A

[1] The adenylpyrophosphatase activity of the enzyme preparation was very slight.

[2] AMP = adenosine-5-phosphate; ADP = adenosine diphosphate.

ratio of acetate to pyrophosphate greater than 2 would indicate that acetoacetate was decomposed by some process other than the phosphoroclastic splitting.

To test this point, the decomposition of acetoacetate was studied in the presence of adenylic acid. Pyrophosphate, acetyl phosphate, and acetate were determined after appreciable decomposition had occurred. The results, presented in Table II, show that almost exactly 2 moles of acetate were formed for each mole of organic phosphate produced. It is almost certain, therefore, that acetoacetate is decomposed only by way of a phosphoroclastic type of reaction (equation (2)).

Factors Influencing Phosphoroclastic Decomposition of Acetoacetate. (a) *Hydrogen Ion Concentration*—The phosphoroclastic decomposition of acetoacetate was studied at pH levels varying from 5.3 to 8.0. Since 2

TABLE II

Decomposition of Acetoacetate in Presence of Adenylic Acid

18 mg. of dry cell-free extract, 24 μM of adenylic acid, and 100 μM of acetoacetate in 1.0 ml. of 0.1 M inorganic phosphate (pH 5.75) were incubated *in vacuo* in a Thunberg tube for 6 hours at 26°.

Increase in 7 min. P	Final acetyl P	Organic P, total*	Final acetate	$\dfrac{\text{Acetate}}{\text{Organic P}}$
μM	μM	μM	μM	
12.4	1.7	14.1	26.7	1.89

* Acetyl phosphate + 7 minute-hydrolyzable phosphate.

moles of steam-volatile acid (acetyl phosphate + acetate) are produced in the phosphoroclastic decomposition of 1 mole of acetoacetate (Table I), the increase in volatile acids was used to measure the reaction.[3] The data from a typical experiment are summarized in Fig. 2. The phosphoroclastic decomposition is greatest in the relatively narrow range of pH 5.7 to 6.2. Decomposition at pH 5.4 and 7.0 is only about one-half as great as at pH 6.0.

(b) *Acetate Concentration*—Since acetate is a product of acetoacetate decomposition, the rate and extent of such a breakdown should be influenced by the acetate concentration. To obtain information regarding this effect, the decomposition of acetoacetate was studied over a wide range

[3] Total acid production is a more reliable measure of phosphoroclastic decomposition than is acetyl phosphate formation, since the hydrolysis of acetyl phosphate, spontaneously and by enzyme action, is also influenced by pH. The use of acid production as a measure of phosphoroclastic decomposition also obviates the necessity of correcting for the relatively slight spontaneous decomposition of acetoacetate to acetone and CO_2.

of acetate concentrations. It was found (Fig. 3) that increasing the concentration of acetate from 0.002 to 0.062 M caused a progressive decrease in acetoacetate decomposition as measured by the formation of acetyl phosphate.[4] At 0.062 M concentration the amount decomposed was only about 25 per cent as great as that decomposed at 0.002 M. An inverse proportionality exists between the logarithm of the initial rate of acetyl phosphate formation and the acetate concentration (Fig. 4).
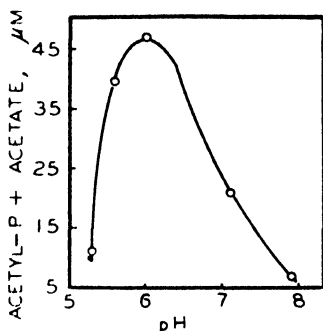
FIG. 2.
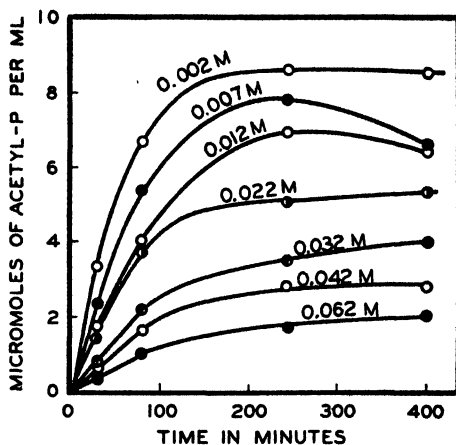
FIG. 3.

FIG. 2. The effect of pH on the phosphoroclastic splitting of acetoacetate. Each Thunberg tube contained 300 μM of acetoacetate, 0.1 M phosphate, and 75 mg. of dry cell-free extract (Lot G). The total liquid volume was 3.0 ml. Temperature, 26°. The abcissa refers to the initial pH; the ordinate to the increase in volatile acids per 2.0 ml. after 320 minutes incubation.

FIG. 3. The effect of acetate concentration on the decomposition of acetoacetate. Each Thunberg tube contained 113 μM of acetoacetate, 102 μM of inorganic phosphate (pH 5.7), 20 mg. of enzyme extract (Lot D), and the indicated amounts of sodium acetate. The total liquid volume was 2.0 ml. The tubes were evacuated and incubated at 26°.

The effect of acetate observed here could be due to (a) a shift in the equilibrium of reaction (2) or (b) to some sort of direct or indirect inhibition of the enzyme system. The first possibility appeared unlikely, since there was a wide variation (4-fold) in the calculated equilibrium constant (equation (2)) for samples incubated at the various acetate levels. Fur-

[4] The final ratio of acetyl phosphate produced to the increase in volatile acid (acetyl phosphate + acetate) was approximately the same (0.26 to 0.33) at all acetate levels. Therefore, the formation of acetyl phosphate can be used as an approximate measure of the relative rate of phosphoroclastic decomposition of acetoacetate under the conditions of this experiment.

thermore, tracer experiments, to be described shortly, have shown that the phosphoroclastic decomposition of acetoacetate is practically irreversible. This discovery proves definitely that the effects of pH and of ace-
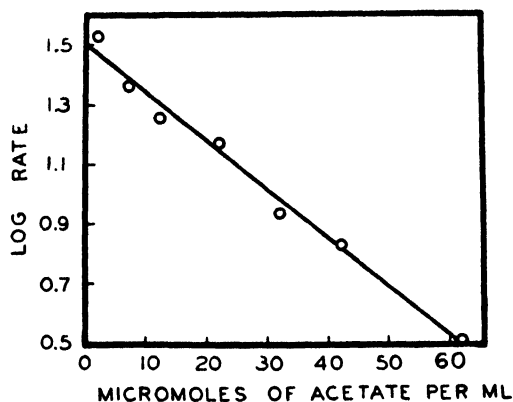
FIG. 4. The effect of acetate concentration on the rate of acetoacetate decomposition. The conditions were the same as in Fig. 3. The rate refers to the micromoles of acetyl phosphate $\times$ 10 produced per ml. in 30 minutes.

TABLE III

Conversion of Acetyl Phosphate to β-Keto Acid

Each Thunberg tube contained 28 μM of acetyl phosphate (initially), 20 mg. of dried cell-free extract (Lot C), and the indicated amounts of sodium acetate and Ba^{++}. The total liquid volume was 2.0 ml. All samples were incubated 240 minutes at 26°.

Sample No.	Sample	Acetyl P*	β-Keto acid†
		μM	μM
1	Control, no enzyme	6.0	
2	" boiled enzyme	5.0	0.2
3	Enzyme, no added acetate‡	11.7	1.1
4	" 40 mg. acetate	13.3	1.3
5	" 80 " "	13.8	
6	" 80 " " 80 mg. Ba^{++}	22.4	0.3

* Determined by the hydroxamic acid method.
† Determined by the aniline acetate method.
‡ Actually 5 μM of acetate were present; the enzyme preparation contained 2 μM and the acetate phosphate preparation 3 μM.

tate are not mass action effects, and, therefore, must be due to some sort of inhibition.

Chemical Studies on Reversibility of Acetoacetate Decomposition. (a) Formation of β-Keto Acid from Acetyl Phosphate and Acetate—If the de-

composition of acetoacetate is a readily reversible reaction, it should be possible to achieve a synthesis of acetoacetate from acetyl phosphate and acetate. Further, if the synthetic reaction occurs to any appreciable extent, it could be detected by measuring the disappearance of acetyl phosphate. In Table III are presented the results of an experiment that shows that enzyme preparations of *C. kluyveri* do catalyze the disappearance of acetyl phosphate and that small but significant amounts of a β-keto acid are produced. The loss of acetyl phosphate is much greater than the keto acid formed. Thus in Sample 3, Table III, the enzyme catalyzed the disappearance of about 6 μM of acetyl phosphate but only 1.1 μM of keto acid were formed. It has been found by later experiments that most if not all of the loss of acetyl phosphate observed under the above conditions is due to enzymatic hydrolysis catalyzed by phosphatases present in the cell-free extract.

The failure to observe a greater accumulation of a keto acid may be due to an unfavorable equilibrium. According to calculations of Lipmann (15) the ΔF^0 for the conversion of acetyl phosphate and acetate to acetoacetate (equation (2)) is about $+1$ kilocalorie at pH 7.0.

Attempts to increase the keto acid formation by increasing the acetate concentration were not very successful (see Samples 3, 4, and 5 of Table III). Also unsuccessful was an attempt to increase the yield of keto acid by the addition of Ba^{++} to precipitate the phosphate that was expected to be liberated in the condensation reaction. Ba^{++} actually retarded rather than stimulated keto acid synthesis. It was found, however, that Ba^{++} did stimulate the disappearance of acetyl phosphate (Sample 6, Table III). The latter effect of Ba^{++} may be due to a stimulation of acetylphosphatase action, either by way of a direct ion effect on the enzyme or by the removal of inorganic phosphate. The latter possibility would seem reasonable in view of the fact that an inhibitory effect of inorganic phosphate on animal acetylphosphatase has been described by Lipmann (15). However, with the enzyme preparations of *C. kluyveri*, it was found that the rate of hydrolysis of acetyl phosphate in 0.05 M phosphate buffer was not significantly different from the rate when no phosphate was added. The exact nature of the Ba^{++} effect is therefore still undetermined.

Other experiments, that will not be described in detail here, have shown that the addition of HCN (0.002 M) causes a greater loss of acetyl phosphate (30 per cent) and a greater accumulation of the keto acid, probably as the cyanohydrin. This effect is being investigated further and will be considered in more detail at another time.

(*b*) *Reduction of Acetoacetate to β-Hydroxybutyrate*—Since only relatively small amounts of the β-keto acid were produced in the above experiments,

no direct attempt was made to obtain an unequivocal identification of the substance. However, since acetoacetate was readily decomposed to acetyl phosphate and acetate (Table I), it appeared likely that the β-keto acid was actually acetoacetate and that it might be the primary oxidant in butyrate synthesis (equation (1)).
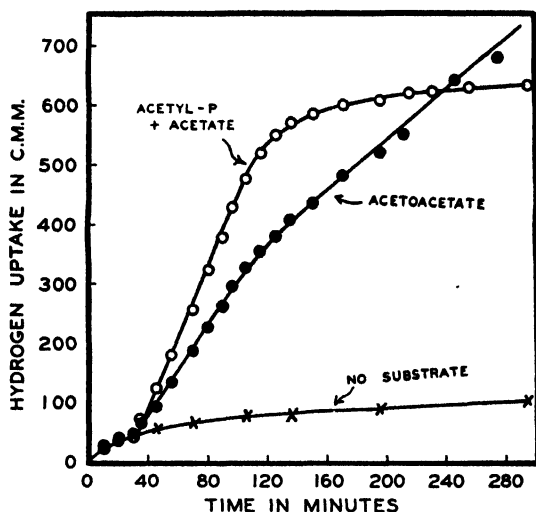
FIG. 5. The reduction of acetoacetate with molecular hydrogen. Each Warburg vessel contained veronal buffer 0.04 M (pH 8.1), 20 mg. of cell-free extract (Lot D), and acetoacetate (69 μM) or acetate (18 μM) + acetyl phosphate (14 μM). The substrates were mixed with the enzyme after 30 minutes. The samples were incubated in H_2 at 26°.

TABLE IV

H_2 Reduction of Acetoacetate

Substrate	−H_2	Δ butyrate
	μM	μM
Acetoacetate, 69 μM	29	2
Acetyl P, 14 μM, + acetate, 18 μM....	24	9.8

See Fig. 5 for experimental details. The data have been corrected for the H_2 uptake (4.4 μM) and the butyrate (2 μM) in a control sample to which no substrate was added.

It has been shown that in a hydrogen atmosphere acetyl phosphate and acetate are readily reduced to butyrate (18). If acetoacetate is the β-keto acid formed in the above experiments and if it is a normal intermediate in butyrate synthesis (equation (1)), then it should be reduced to butyrate in a hydrogen atmosphere. The results of an experiment to

test this point are given in Fig. 5, which shows that acetoacetate was reduced; however, the rate of reduction was not as great as that obtained with a mixture of acetyl phosphate and acetate. The latter fact was inconsistent with the view that acetoacetate is an intermediate. Therefore, the volatile acid fraction was examined to determine whether butyrate had been produced. The data of Table IV show that, although 29 μM of hydrogen were consumed, only 2 μM of butyrate were formed. It is obvious, therefore, that most of the acetoacetate had been reduced to some other substance.

In the above experiment, a relatively high concentration of acetoacetate (69 μM) was added and the experiment was discontinued before the H_2 uptake was complete. It could be argued, therefore, that the failure to obtain more butyrate was due to the fact that the hydrogen-activating system was limiting and that the primary reduction of acetoacetate to some intermediate stage (viz. β-hydroxybutyrate) was favored, but that a further reduction to butyrate might have taken place if the reduction had been carried to completion. To exclude this possibility, other experiments were made with relatively low acetoacetate levels, and were continued until no more hydrogen was consumed. It was found that only 1 mole of hydrogen was consumed per mole of acetoacetate added. The addition of more acetoacetate to the enzyme mixture after the H_2 uptake had stopped resulted in a further uptake, until again 1 mole of hydrogen was consumed per mole of additional acetoacetate. Since 2 moles of hydrogen are required to reduce 1 mole of acetoacetate to butyrate, these results indicated that acetoacetate was reduced to some substance in an intermediate state of oxidation. In preparations from animal tissue acetoacetate is reduced to β-hydroxybutyrate (6, 9); therefore, it seemed a reasonable possibility that this compound was the reduction product of acetoacetate in the above experiments. Accordingly, analyses for β-hydroxybutyrate were made on the various enzyme mixtures. The results are presented in Table V. For each mole of acetoacetate added, 1 mole of hydrogen was consumed and almost 1 mole of β-hydroxybutyrate was formed. These data show quite definitely that β-hydroxybutyrate is the final end-product of acetoacetate reduction (equation (6)).

$$CH_3COCH_2COO^- + H_2 \rightarrow CH_3CHOHCH_2COO^- \tag{6}$$

In view of these results, it seemed very unlikely that acetoacetate is a precursor of butyrate. The possibility remained, however, that acetoacetate, though not an actual intermediate, is nevertheless closely related to the true intermediate. For example, a phosphorylated derivative might be involved. Attempts to obtain a reduction of acetoacetate beyond the β-hydroxybutyrate stage by the addition of phosphorylating agents such as

inorganic phosphate, acetyl phosphate, or adenosine triphosphate were completely unsuccessful. It is worth noting that the rate of acetoacetate reduction was not influenced by the presence of inorganic phosphate or arsenate (0.025 M).

Of particular interest were experiments in which the reduction of mixtures of acetyl phosphate, acetate, and acetoacetate was studied. The results of one such experiment are presented in Fig. 6. The data show that the rate of reduction of a mixture of acetyl phosphate, acetate, and acetoacetate was almost exactly the same as the sum of the rates observed for a mixture

TABLE V

Reduction of Acetoacetate to β-Hydroxybutyrate

Each Warburg vessel contained 0.04 M veronal buffer, 20 mg. of cell-free extract (Lot D), and the indicated amounts of acetyl phosphate and acetoacetate. The total liquid volume was 2.0 ml. The samples were incubated (26°) in a H_2 atmosphere until no further hydrogen uptake occurred (5 hours).

Acetoacetate, initial	Acetyl P, initial	$-H_2$	Acetoacetate, final*	Δ aceto-acetate	Δ β-hydroxy-butyrate*
μM	*μM*	*μM*	*μM*	*μM*	*μM*
21	0	22	1.7	19.3	18.6
21	0	22	1.6	19.4	23.0
9.5	0	10.5			7.6
9.5	9.4	21.5			7.4
0	·0	0†	0.46		0.35

* Estimated by the method of Behre and Benedict (2, 3). The experimental values were multiplied by the factor 1.33 to correct for incomplete recovery of the β-hydroxybutyrate as acetone (20).

† The data for H_2 uptake have been corrected for the H_2 consumed (4.4 $μM$) in the sample containing no added substrate.

of acetyl phosphate and acetate, and of acetoacetate alone. This result indicates that the enzyme involved in the reduction of acetyl phosphate and acetate to butyrate is different from that which catalyzes the reduction of acetoacetate. It is quite clear, therefore, that acetoacetate is not a normal intermediate in the synthesis of butyrate.

In view of the fact that acetoacetate is readily decomposed to acetyl phosphate and acetate, it may seem strange that in the foregoing experiments acetyl phosphate and acetate were reduced to butyrate, while acetoacetate was not. In this connection it should be pointed out that all of the above experiments were done at pH 8.0. At this pH the reduction with H_2 is rapid, whereas the phosphoroclastic splitting of acetoacetate does not occur.

Tracer Studies on Reversibility of Phosphoroclastic Decomposition of

Acetoacetate—The above experiments show that acetoacetate is readily reduced to β-hydroxybutyrate in a hydrogen atmosphere and that the enzyme catalyzing this reduction apparently is not identical with that catalyzing the reduction of acetyl phosphate and acetate to butyrate. Therefore, if acetyl phosphate and acetate are readily converted to acetoacetate, one would expect that the incubation of these substances in a hydrogen atmosphere would lead to the accumulation of appreciable
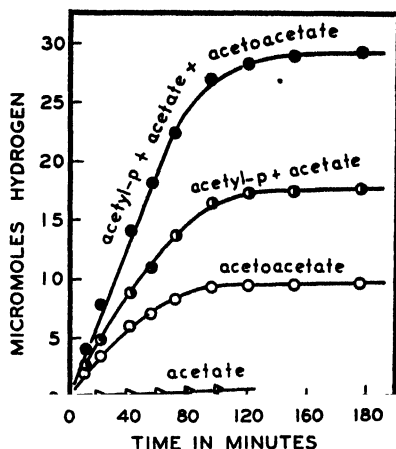
Fig. 6. The reduction of acetoacetate and acetyl phosphate by H_2. Each Warburg vessel contained 0.03 M veronal buffer (pH 8.1), 20 mg. of cell-free extract (Lot D), 150 μM of acetate, and where indicated 10 μM of acetoacetate and 10 μM of acetyl phosphate. The total liquid volume was 2.0 ml. The gas phase was H_2. The substrates were dumped from the side arms after the enzyme preparation was saturated with H_2 (about 50 minutes).

amounts of β-hydroxybutyrate. This did not occur. In the reduction of acetyl phosphate and acetate, practically no β-hydroxybutyrate was formed. Instead, there was an almost quantitative conversion of the acetyl phosphate to butyrate (18).

These results indicate that the phosphoroclastic decomposition of acetoacetate is not rapidly reversible. There remains the possibility, however, that acetyl phosphate and acetate are converted to an as yet unidentified C_4 compound, which under highly reducing conditions is preferentially reduced to butyrate, but which in the absence of a hydrogen donor (H_2) is converted to acetoacetate.

$$\text{Acetyl phosphate} + \text{acetate} \leftrightarrows \text{Compound } c \leftrightarrows \text{acetoacetate}$$
$$\downarrow H_2$$
$$\text{Butyrate}$$
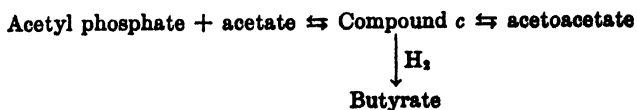
In view of these considerations, the reversibility of the phosphoroclastic decomposition of acetoacetate was studied by the isotope technique.

Phosphoroclastic Decomposition of Acetoacetate in Presence of C^{14}-Labeled Acetate—Acetate equally labeled in both the methyl and carboxyl groups with C^{14} was incubated in an evacuated Thunberg tube with the enzyme and unlabeled acetoacetate in the absence of a hydrogen donor. Samples were adjusted to pH 6.5, at which the phosphoroclastic decomposition of acetoacetate is rapid, and to pH 8.1, at which little or no decomposition of

TABLE VI

Incubation of Acetoacetate with Doubly Labeled Acetate

Each Thunberg tube contained 50 mg. of dried cells (Lot I), 200 μM of inorganic phosphate, acetate equally labeled in the methyl and carboxyl group with C^{14} (17,600 counts per minute), and the indicated amounts of acetoacetate, acetate, and acetyl P. The samples were incubated at 26° for 70 minutes.

Sample No.	pH	Acetoacetate		Acetate	Acetyl P		C^{14} of acetoacetate after incubation, counts per min. per microatom C		
		Initial	Final	Initial	Initial	Final	Theo-retical*	Carboxyl group†	2, 3, 4 carbons (average‡)
		μM	μM	μM	μM	μM			
1	6.5	135	121	37	32	§	26	0.13	0.17
2	6.5	135	103	9	0	25.4	31	0.8	§
3	8.1	157	181	37	32	21	20	0.19	0.06

* Calculated on the assumption that there was a complete equilibration between the acetate, acetyl P, and acetoacetate. $\overset{*}{C}H_3COOPO_3^- + \overset{*}{C}H_3COO^- \leftrightarrows \overset{*}{C}H_3CO\overset{*}{C}H_2\overset{*}{C}OO^- + HPO_4^-$.

† Determined by measuring the radioactivity of the CO_2 evolved by decomposition of acetoacetate with aniline acetate.

‡ Estimated by measuring the radioactivity of the 2,4-dinitrophenylhydrazone of the acetone liberated when acetoacetate is decomposed with aniline acetate.

§ Not determined.

acetoacetate occurs (Fig. 2), but where acetyl phosphate and acetate are readily reduced to butyrate. Also, since acetate apparently inhibits the decomposition of acetoacetate, samples were prepared with both a relatively high and a low acetate level. After incubation, the acetoacetate was decomposed by the addition of aniline acetate and the C^{14} content of the carboxyl carbon atom and of the α-, β-, and γ-carbon atoms was determined as previously described.

The results (Table VI) show that only very small amounts of isotopic carbon were incorporated into acetoacetate. At the higher acetate level (Samples 1 and 3) the isotope content of the residual acetoacetate was less

than 1 per cent of that theoretically possible, assuming that complete equilibrium existed among acetoacetate, acetyl phosphate, and acetate.[5] A somewhat greater C^{14} content (2.6 per cent of theoretical) was found in the acetoacetate of Sample 2, Table VI, to which only a small amount of acetate and no acetyl phosphate were added initially. The formation of acetyl phosphate (25 μM) during the incubation of Sample 2 reflects a comparable decrease in the concentration of acetoacetate and shows that the enzyme responsible for the phosphoroclastic decomposition of acetoacetate was active.

The small but significant amounts of isotopic carbon found in both the carboxyl group and in the carbonyl residue of the acetoacetate show that a slight conversion of acetate to acetoacetate did occur. Nevertheless,

TABLE VII

Oxidation of Carboxyl-Labeled Butyrate in Presence of Acetoacetate

The Warburg vessel contained 0.1 M inorganic phosphate (pH 8.1), 50 mg. of dried cells (Lot I), and the indicated amount of acetoacetate and carboxyl-labeled butyrate. The sample was shaken in air at 26° until oxygen uptake had ceased (85 minutes).

Carboxyl-labeled butyrate, initial	C^{14} content of butyrate added	O_2 uptake	Acetoacetate*		C^{14} in carboxyl group of final acetoacetate†
			Initial	Final	
μM	*counts per min. per μM*	μM	μM	μM	*counts per min. per μM*
37	1390	38	157	135	0.18

* Determined manometrically by aniline acetate decomposition (11).

† Determined by measuring the radioactivity of CO_2 evolved when acetoacetate is decomposed with aniline acetate.

the obvious conclusion is that the phosphoroclastic decomposition of acetoacetate to acetyl phosphate and acetate is not readily reversible.

Oxidation of Carboxyl-Labeled Butyrate in Presence of Acetoacetate—The foregoing experiment proves that acetyl phosphate and acetate are not readily converted to acetoacetate and thus confirms the previous conclusion that acetoacetate is not an intermediate in the synthesis of butyrate. The possibility still remained, however, that the oxidation of butyrate occurred by a path which was not identical with that involved in the synthesis of butyrate, and that acetoacetate might therefore be an inter-

[5] A rapid equilibration of acetate and acetyl phosphate is indicated by results obtained in isotope experiments on the synthesis of butyrate from ethanol and labeled acetate (1), and by the observation that a rapid transphosphorylation occurs between acetyl phosphate and propionate (unpublished results, Stadtman and Barker).

mediate in the oxidation of butyrate. To test this possibility an experiment was made in which butyrate, labeled in the carboxyl group with C^{14}, was oxidized in the presence of acetoacetate. When oxidation of the butyrate was complete, the residual acetoacetate was decomposed with aniline acetate and the isotope content of the carboxyl group was measured as in the preceding experiments. The results (Table VII) show that, although the butyrate was completely oxidized to acetyl phosphate and acetate (1 mole of oxygen was consumed per mole of butyrate added (17)), practically none of the C^{14} entered the acetoacetate. This proves conclusively that acetoacetate is not a normal intermediate in the oxidation of butyrate.

<div align="center">DISCUSSION</div>

The phosphoroclastic decomposition of acetoacetate to acetyl phosphate and acetate, previously postulated by Lipmann (15), has now been shown to be catalyzed by enzyme preparations of *C. kluyveri*. The reverse reaction, *i.e.* the conversion of acetyl phosphate and acetate to acetoacetate, was found not to occur to any appreciable extent. Thus only trace quantities of a β-keto acid were produced when acetyl phosphate and acetate were incubated with the enzyme preparations. The relatively non-reversible nature of the reaction was further demonstrated by the fact that during the phosphoroclastic decomposition of acetoacetate in the presence of labeled acetate scarcely any of the isotopic carbon was incorporated into the acetoacetate.

The situation is therefore the reverse of that encountered in animal systems where it has been found that acetate, in the presence of a suitable oxidizable substrate such as pyruvate (13) or a fatty acid (14, 22), is readily converted into acetoacetate, whereas the reverse splitting of the acetoacetate to acetate occurs to a very limited extent (12) or not at all (4).

Since it has been shown that acetyl phosphate and acetate are normally reduced to butyrate by the enzyme preparations of *C. kluyveri*, the failure to obtain a synthesis of acetoacetate from these substrates can be regarded as evidence that acetoacetate is not a normal intermediate in the synthesis of butyrate. This conclusion is further verified by the finding that acetoacetate can be reduced only as far as β-hydroxybutyrate under conditions in which acetyl phosphate and acetate are reduced all the way to butyrate. Furthermore, evidence is presented showing that different enzymes are involved in the reduction of acetyl phosphate and acetate to butyrate and of acetoacetate to β-hydroxybutyrate.

The precise manner in which acetoacetate is decomposed to acetyl phosphate and acetate is still undetermined. It may be decomposed directly to these substances or it may first be converted to a 4-carbon compound

(Compound c, Scheme I) on the main pathway to butyrate synthesis. In either event the conversion is relatively irreversible.

The reduction of acetoacetate to β-hydroxybutyrate, as shown by Scheme I, is apparently off the main pathway of butyrate synthesis, and in this respect is comparable to the corresponding reduction observed in animals (6, 9, 10).

$$\text{Acetyl P + acetate} \underset{\longleftarrow}{\rightleftharpoons} \text{Compound } c \xrightarrow[\longleftarrow]{+4H} \text{butyrate} + H_3PO_4 \tag{I}$$

$$\text{acetoacetate} \xrightarrow{+2H} \beta\text{-hydroxybutyrate}$$

Since the phosphoroclastic decomposition of acetoacetate is essentially irreversible, it is difficult to account for the fact that the decomposition is incomplete. Generally, the decomposition ceases long before the substrate is used up. It has been shown that the hydrogen ion concentration and the acetate concentration are among the factors influencing the extent of decomposition. Beyond this, however, nothing is known about the nature of the inactivation involved. One explanation that seems feasible for the moment is that the acetyl phosphate and acetate produced in the decomposition of acetoacetate recombine to form another 4-carbon compound that competes with the acetoacetate for the enzyme involved in the decomposition of the latter. That a 4-carbon compound other than acetoacetate (corresponding to Compound c in Scheme I) is produced from acetyl phosphate and acetate appears probable in view of the fact that these substances are reduced to butyrate. Whether or not this postulated substance can effectively compete with acetoacetate for the enzyme causing the decomposition of the latter has yet to be demonstrated.

SUMMARY

It has been shown that enzyme preparations of *Clostridium kluyveri* catalyze the phosphoroclastic decomposition of acetoacetate to acetyl phosphate and acetate.

When the phosphoroclastic decomposition is allowed to occur in the presence of doubly labeled acetate ($C^{14}H_3C^{14}OONa$), practically no isotopic carbon is incorporated into the acetoacetate. The decomposition of acetoacetate appears therefore to be essentially irreversible.

When carboxyl-labeled butyric acid is oxidized completely to acetyl phosphate and acetate in the presence of inactive acetoacetate, almost no isotopic carbon is incorporated into the acetoacetate. Therefore, acetoacetate is not an intermediate in the oxidation of butyrate.

In a hydrogen atmosphere, acetoacetate is reduced almost quantitatively to β-hydroxybutyrate; little or no butyrate is formed. Under these same

conditions acetyl phosphate and acetate are reduced all the way to butyrate. Acetoacetate therefore cannot be an intermediate in the synthesis of butyrate by *C. kluyveri*.

BIBLIOGRAPHY

1. Barker, H. A., Kamen, M. D., and Bornstein, B. T., *Proc. Nat. Acad. Sc.*, **31**, 373 (1945).
2. Behre, J. A., *J. Biol. Chem.*, **136**, 25 (1940).
3. Behre, J. A., and Benedict, S. R., *J. Biol. Chem.*, **70**, 487 (1926).
4. Buchanan, J. M., Sakami, W., Gurin, S., and Wilson, D. W., *J. Biol. Chem.*, **159**, 695 (1945).
5. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M., and Yankwich, P. F., Isotopic carbon, New York (1949).
6. Dakin, H. D., *J. Biol. Chem.*, **8**, 97 (1910).
7. Davies, R., *Biochem. J.*, **36**, 589 (1942).
8. Davies, R., *Biochem. J.*, **37**, 230 (1943).
9. Green, D. E., Dewan, J. G., and Leloir, L. E., *Biochem. J.*, **31**, 934 (1937).
10. Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2143 (1935).
11. Jowett, M., and Quastel, J. H., *Biochem. J.*, **29**, 2159 (1935).
12. Lehninger, A. L., *J. Biol. Chem.*, **143**, 147 (1942).
13. Lehninger, A. L., *J. Biol. Chem.*, **161**, 413 (1945).
14. Lehninger, A. L., and Kennedy, E. P., *J. Biol. Chem.*, **173**, 753 (1948).
15. Lipmann, F., *Advances in Enzymol.*, **6**, 231 (1946).
16. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **180**, 1085 (1949).
17. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **180**, 1095 (1949).
18. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **180**, 1117 (1949).
19. Swendseid, M. E., Barnes, R. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 47 (1942).
20. Van Slyke, D. D., *J. Biol. Chem.*, **32**, 455 (1917).
21. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 671 (1941).
22. Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **155**, 143 (1944).
23. Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, **158**, 411 (1945)

STUDIES ON THE HAZARD INVOLVED IN USE OF C14

1. RETENTION OF CARBON FROM LABELED SODIUM BICARBONATE*

By HOWARD E. SKIPPER, LOCKE WHITE, Jr., AND CARL E. BRYAN

(*From the Southern Research Institute, Birmingham, Alabama*)

The entrance of carbon from carbon dioxide into both the organic and inorganic metabolic pathways has been well established (1-6).[1] This fact coupled with knowledge of the half life of C14 (approximately 5000 years) has led to considerable concern regarding hazard to persons utilizing this useful isotope. Information as to the degree and duration of tissue exposure following the intake of compounds labeled with C14 is required if the hazard accompanying the use of this isotope is to be properly assessed. The degree and duration of tissue exposure to the β-particles from C14 are dependent on concentration (dosage and distribution) and turnover, both of which are in turn dependent on the carbon-utilizing metabolic system involved and its dynamic state. Tissue exposure data can best be obtained by following the quantitative gross distribution and turnover of C14 from variously labeled inorganic and organic molecules over extended periods.

Bloom, Curtis, and McLean (5) showed by means of autoradiographic techniques that the bones of rats injected with BaC14O3 or NaHC14O3 (75 to 100 μc.) retained activity at 16 weeks. Sections of liver and kidney were reported by the above authors to give fairly intense autoradiographs at 3 days and 2 weeks, but were negative after longer intervals.

Armstrong, Schubert, and Lindenbaum (6) in studies of the distribution of radioactive carbon administered as carbonate have measured the rate of excretion in the expired air, urine, and feces and the retention in various organs, bones, and teeth at 6 days and in certain important chemical constituents at 6 and 40 days.

Brues and Buchanan (7) investigated the over-all CO2 metabolism of tissues and animals and observed that inhaled C14O2 is absorbed very efficiently and is lost at a rate comparable to that of its intake. They suggest that such an equilibration rate probably holds with regard to

incorporation of $C^{14}O_2$ in compounds in a steady state, but not in the case of growing tissue or when exposure is continuous over a long period.

The present experiments, although repetitious in certain respects, have been designed to obtain quantitative data on $C^{14}O_2$ fixation, from which tissue, organ, and body exposure can be calculated if the dosage is known.

<center>EXPERIMENTAL</center>

A radioactive sodium bicarbonate solution was prepared by the method of Evans and Slotin (8), which involved passage of $C^{14}O_2$ liberated from

<center>TABLE I</center>
<center>Rate of Excretion of C^{14} Following Injection of $NaHC^{14}O_3$*</center>

Period	Specific activity of sample†	Per cent of total injected radioactivity recovered	Cumulative per cent of total radioactivity recovered
min.			
0–10	13,770	66	66
10–20	3,603	16	82
20–30	1,750	6.2	88.2
30–60	500	4.6	92.8
hrs.			
1– 2	84	1.3	94.1
2– 4	24.4	0.65	94.75
4– 6	11.2	0.26	95.01
6–12	5.6	0.44	95.45
12–24	4.8	0.59	96.09
24 hr. composite urine sample	115.1	1.35	97.39

* Average data obtained on two 23 gm. male mice, CFW strain.
† Specific activities in microcuries per mole of carbon.

$BaC^{14}O_3$ into an equivalent amount of N sodium hydroxide. The solution thus prepared had a concentration of 2.5 mg. of $NaHC^{14}O_3$ per 0.2 ml. and a specific activity of 600 mc. per mole of carbon. Thus, 0.2 ml. of this solution contained 18 μc. of C^{14}.

Mice were injected intraperitoneally each with 0.2 ml. of this solution and immediately placed in a metabolism chamber. Data on the clearance of C^{14} by the respiratory and the urinary routes are presented in Table I. The details of the procedures used in the collection of expired carbon dioxide and determination of C^{14} content have been described recently (9).

This information on rate of clearance of the principal portion of bicarbonate C^{14} has been plotted along with results obtained by Armstrong

et al. (6), who, using $Na_2C^{14}O_3$, obtained a very similar curve with the rat (Fig. 1).

In order to determine the chemical state of the C^{14} in blood of mice 24 hours after injection with $NaHC^{14}O_3$, blood samples were assayed for
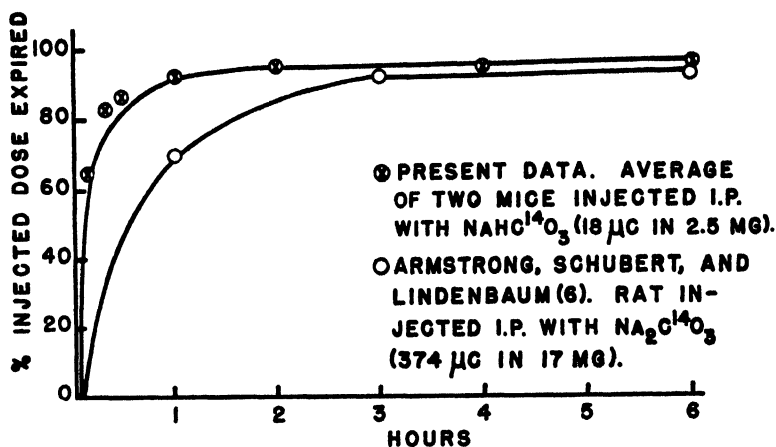
FIG. 1. Rate of loss of $C^{14}O_2$ by the respiratory route

TABLE II

Investigation of Fixed C^{14} Content of Mouse Blood 24 Hours after Injection of $NaHC^{14}O_3$ (18 μc. in 2.5 Mg.)

	Specific activity of blood*		
Animal No.	Untreated	Treated†	Relative activity, treated / untreated
38‡	0.37	0.30	0.81
52	0.57	0.55	0.96
53	2.24	1.94	0.87
54	2.67	2.56	0.96

* Specific activity in microcuries per mole of carbon.

† 1 N acetic acid added to sample, followed by 24 hour vacuum treatment (1 mm. of Hg). This column represents fixed or non-carbonate C^{14}.

‡ Akm mouse with spontaneous leucemia.

radioactivity after acid-vacuum treatment and compared with untreated blood samples. These data are given in Table II.

In studies designed to provide information regarding the degree and duration of tissue exposure following administration of $NaHC^{14}O_3$, activity assays have been carried out on mice 24 hours, 48 hours, 1 week, 2 weeks, 4 weeks, and 3 months after injection. The mice used in these

experiments were adult CFW strain animals weighing about 25 gm. and about 6 weeks of age at the beginning of an experiment. This strain is past its period of rapid weight gain at 6 weeks. The animals were injected at the level of 18 μc. each and maintained in a metabolism chamber with food and water until sacrificed for distribution assays. Tissues selected for study were blood, spleen, kidney, liver, lungs, brain, muscle,

TABLE III

Specific Activity* of Tissues and Bone at Various Periods after Injection of NaHC¹⁴O₃ (18 μc.)

Tissue	Period after injection					
	24 hrs.	48 hrs.	1 wk.	2 wks.	4 wks.	12 wks.
Blood........	1.09	0.40	0.25	0.35	0.11	0.10
Spleen	0.73	0.60		0.02	0.414	0.19
Liver....	1.11	0.87	0.48	0.13	0.02	0.07
Kidney..	0.78	0.83	0.21	0.16	0.05	0.01
Lungs.	0.57	0.56	0.40	0.09	0.09	0.02
Brain...	0.26	0.67	0.18	0.22	0.13	0.03
Jejunum . ..	2.16	0.97	0.18	0.11	0.02	
Muscle	0.75	0.31	0.27	0.03	0.07	0.05
Skin and hair† . ..	0.21	0.49	0.55	0.15	0.14	0.08
Long bones	1.01	0.81	0.33	0.28	0.11	0.10

* Specific activity in microcuries per mole of carbon.
† Erratic data probably due to early high activity urine contamination.

TABLE IV

Specific Activity of Whole Carcass and Fetus of Mouse 24 Hours after Injection with NaHC¹⁴O₃ (18 μc.)

Sample	Specific activity
	microcuries per mole of C
Carcass of mouse.....................................	0.41
" " " (acid-treated)........................	0.45
Embryos...	1.50
" (acid-treated)...............................	2.04

jejunum, skin and hair, and long bones. A summary of the specific activities of these tissues at various intervals after injection of NaHC¹⁴O₃ is presented in Table III. All activity measurements were made with use of tissue oxidation and gas phase counting techniques recently described (9).

One simple experiment has been carried out comparing the C¹⁴ fixation

of slow *versus* rapidly growing tissue. A 4 month-old pregnant Akm mouse (13 days after conception) was injected with 18 μc. of C^{14} as $NaHC^{14}O_3$. 24 hours after injection the animal was taken from the metabolism chamber, sacrificed and skinned, and the embryos (six in number, weighing a total of 1.53 gm.) were removed. The whole carcasses of the mouse and the embryos were homogenized separately in a Waring blendor and representative samples of each assayed for activity. Additional samples of both homogenates were treated with a few drops of 1 M HCl, submitted to vacuum to remove any C^{14} bicarbonate, and then assayed for radioactivity. The results of this experiment are given. in Table IV.

DISCUSSION

From the data presented in Fig. 1 it appears that greater than 90 per cent of the CO_2-bicarbonate system carbon is turned over in 3 hours. It is interesting to note that with use of a different animal and a much lower activity, we have obtained a rate of expiration of $C^{14}O_2$ curve somewhat similar to that reported by Armstrong *et al.* (6). It is not surprising to observe that at 24 hours after injection of $NaHC^{14}O_3$ carbon 14 is retained in the blood fixed in an organic form. Data from this laboratory and many others have demonstrated that $C^{14}O_2$ enters into fat, protein, and carbohydrate metabolism.

The dynamic principles involved in carbon turnover previously mentioned by Brues and Buchanan are illustrated in the present data. From Table III it can be seen that jejunum, a rapidly proliferating tissue, picks up and loses carbon from $NaHC^{14}O_3$ more rapidly than muscle, which is, from the mitotic standpoint, in a more steady state, and the rapidly growing embryonic tissue exhibited a more rapid uptake of C^{14} than did the mature parent mouse tissue (Table IV).

From the information presented in Table III, some assumptions regarding the average mass of certain mouse tissues and fluids (Table V), and the percentage carbon of each sample (determined gravimetrically), it is possible to calculate the approximate total activity contained in the various organs and tissues studied (Table VI). These body retention data are plotted in Fig. 2. If the amount of C^{14} in the body or a portion thereof and the average energy of radiation of this isotope are known, it is simple to convert tissue or total body activities into Roentgen units or rep[2] (the basis for radiation tolerance calculations). With the assumption of even distribution of the isotope, such calculations have been performed regarding total body radiation (Table VII).

Calculations on the total body radiation from fixed C^{14} derived from

[2] 1 rep or roentgen-equivalent-physical = 83 ergs per gm. of tissue.

NaHC¹⁴O₃ tend to minimize the hazard to the experimenter utilizing this isotope. The rather sweeping assumption, made in these calculations,

TABLE V

General Assumptions on Average Mass of Mouse Organs and Tissues Used

Tissue	Per cent of total body weight	Tissue	Per cent of total body weight
Blood*..	6.4	Brain†	1.8
Spleen†	0.9	Gastrointestinal tract‡	10.0
Liver†	6.0	Muscle*..	41.5
Kidney†	1.6	Skin and hair†	13.4
Lungs†	0.7	Skeleton*	17.1

Actual organ or tissue weights have been used in all calculations except in the case of blood, gastrointestinal tract, muscle, and skeleton.

* Extrapolated from man to mouse.

† Actual data from a large number of autopsies, this laboratory.

‡ Data from small number of autopsies, this laboratory.

TABLE VI

Total Activities of Tissues and Bone (in Millimicrocuries) at Various Periods after Injection of NaHC¹⁴O₃ (18 μc.)

Tissue	Period after injection					
	25 hrs.	48 hrs.	1 wk.	2 wks.	4 wks.	12 wks.
Blood...............	11.8	4.1	2.9	3.8	1.2	1.4
Spleen.	1.0	1.5		0.1	1.0	0.5
Liver..	18.9	11.6	7.9	3.4	0.5	1.4
Kidneys	2.5	3.4	1.1	0.8	0.1	0.05
Lungs....	0.8	1.0	0.8	0.1	0.2	0.04
Brain.....................	0.9	2.6	0.8	1.0	0.6	0.1
Gastrointestinal tract........	53.6	23.8	3.8	2.2	0.4	
Muscle.....................	99.6	40.1	37.5	4.3	7.1	6.7
Skin and hair..............	11.9	41.0*	41.3*	7.7	8.4	6.0
Skeleton....................	44.8	39.8	16.2	13.1	4.7	4.9
Total activity..............	245.8	168.9	112.3	36.5	24.2	21.1
% of total dose injected....	1.37	0.94	0.62	0.20	0.13	0.12

18 μc., if all retained, would give a total energy of 4600 ergs per day (1 rep = 0.083 ergs per mg.); (4600/0.083) = 55,420 r. (total possible); (55,420/25,000) = 2.22 rep.

* These figures appear to be out of line, probably because of dried urine contamination.

of even distribution of C¹⁴ throughout the body has some basis in fact from a gross anatomical standpoint for the periods studied (Table III), but further investigations are required to rule out the possibility of selec-
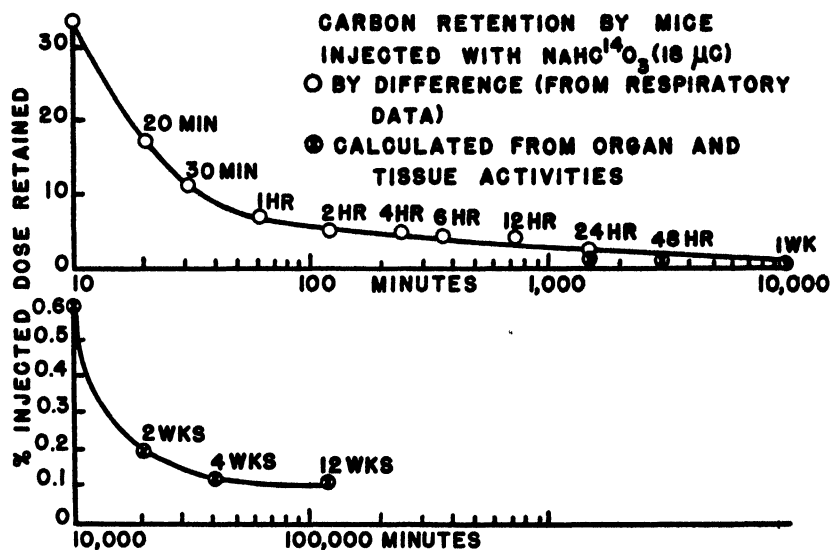
FIG. 2. C^{14} retention by mice injected with $NaHC^{14}O_3$

TABLE VII

Body Radiation of Mice Injected with $NaHC^{14}O_3$ (18 μc.)

Period	Body C^{14} content (integrated)		Body radiation	
	Microcuries	Per cent of injected dose	Total rep per period	Average daily rep per period
0–60 min.	4.3	23.6	0.0250	
1–2 hrs.	1.12	6.2	0.0058	
2–4 "	1.00	5.6	0.0104	
4–6 "	0.92	5.12	0.0090	
6–12 "	0.86	4.77	0.0266	
12–24 hrs.	0.54	2.93*	0.0333	
0–24 "	0.87	4.80	0.110	0.110
24–48 "	0.21	1.15	0.053	0.027
1–7 days	0.18	1.00	0.1328	0.022
7–14 "	0.07	0.41	0.0636	0.009
2–4 wks.	0.03	0.16	0.0496	0.004
4–12 "	0.02	0.13	0.1613	0.003

Tolerance dose limits for a 24 hour exposure as set by the Clinton Laboratories = 0.1 rep. This dose is considered to produce no known effects to man when exposed indefinitely. Factor of safety probably no more than 2 or 3 (10).

* This value is an average of the 12 hour C^{14} level obtained by difference (100 per cent − per cent expired) and the 1.37 per cent found in the tissues at 24 hours.

tive concentration or slower rate of turnover of C^{14} in the finer structures, *i.e.* cell nuclei, chromosomal fractions, etc., where radiation effects

are especially important. Data are already available showing a slower rate of turnover of C^{14} in bone shaft than in other fractions of the bone (5). Experiments are under way in which C^{14} fixation is being studied in certain cellular elements thought to be related to genetic function. It seems probable too that insoluble carbonates and organic C^{14}, especially in the form of natural metabolites which might enter the metabolic pool before being degraded to C^{14}O$_2$, may present a quite different picture.

Although a small percentage of the C^{14} from sodium bicarbonate was retained over a long period, it can be seen from Table VII that a mouse injected at the level of 18 μc. received the tolerance dose for a 24 hour period (0.1 rep) only during the 1st day after injection. The average daily exposure for the 1st week was of the order of 0.02 rep.

If the difference in weight of a mouse and an average man (25 gm. *versus* 70,000 gm.) is taken into account and a linear relationship between C^{14}O$_2$ fixation and blood bicarbonate specific activity is assumed, it would appear that about 50 mc.[3] of C^{14} injected as NaHC^{14}O$_3$ or breathed in as C^{14}O$_2$ over a short interval would be required to produce a 0.1 rep exposure for a single day in a 70 kilo man. This calculation also assumes the same non-specific distribution and rate of bicarbonate turnover in mice and man. The latter assumption is probably not valid, perhaps requiring some correction downward of the 50 mc. figure. However, the decrease in average daily body radiation as a result of body compound turnover might at the end of a 2 week period provide a 10-fold safety factor which should take care of any species difference in bicarbonate half life. The above figure is *not* suggested as a permissible dose. Brues and Buchanan's (7) tentative maximum permissible retained dose for man of 30 μc. is a reasonable estimate containing certain safety factors. These authors state that in the case of a metabolically inert compound in high local concentration a further reduction of this figure might be necessary.

SUMMARY

1. Studies on the rate of expiration of C^{14}O$_2$ following injection of NaHC^{14}O$_3$ indicated that the biological half life of bicarbonate carbon in mice at the time of injection is less than 10 minutes. Greater than 90 per cent of the total activity injected was lost through the respiratory route in 1 hour.

2. At 24 hours after injection of NaHC^{14}O$_3$ a comparison of the specific activity of untreated and acid-vacuum-treated blood showed that most of the C^{14} in the blood was fixed in a non-carbonate form.

[3] In 2 years of continuous effort and tracer studies on some 75 mice, this laboratory has used but 5 mc. of C^{14} and almost all of this can be accounted for in stored BaCO$_3$ samples.

3. No profound localization of C^{14} from $NaHC^{14}O_3$ has been observed with regard to organs and tissues. However, uptake and turnover were more rapid in the jejunum, a tissue having a high mitotic activity, than in other tissues studied. The specific activity of whole long bones paralleled that of the blood up to 12 weeks. Bloom, Curtis, and McLean have shown localization in the shaft of the bone.

4. When $NaHC^{14}O_3$ was injected into a pregnant mouse, the rapidly growing embryo tissues were found to fix C^{14} more rapidly than the parent mouse tissues.

5. Data have been presented showing the retention of C^{14} from $NaHC^{14}O_3$ in nine tissues, bone, and the total body of mice at 24 hours, 48 hours, 1 week, 2 weeks, 4 weeks, and 12 weeks.

6. The body radiation of mice injected with 18 μc. of $NaHC^{14}O_3$ has been calculated to be 0.11 rep per day for the first 24 hours. The average daily rep dropped to about 0.003 for the period of 1 to 3 months. These calculations were based on the assumption of equal distribution.

BIBLIOGRAPHY

1. Wood, H. G., *Physiol. Rev.*, **26**, 198 (1946).
2. Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159**, 475 (1945).
3. Delluva, A. M., and Wilson, D. W., *J. Biol. Chem.*, **166**, 739 (1946).
4. Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140**, 171 (1941).
5. Bloom, W., Curtis, H. H., and McLean, F. C., *Science*, **105**, 45 (1947).
6. Armstrong, W. D., Schubert, J., and Lindenbaum, A., *Proc. Soc. Exp. Biol. and Med.*, **68**, 233 (1948).
7. Brues, A. M., and Buchanan, D. L., *Cold Spring Harbor Symposia Quant. Biol.*, **13**, 52 (1948).
8. Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **141**, 439 (1941).
9. Skipper, H. E., Bryan, C. E., White, L., Jr., and Hutchison, O. S., *J. Biol. Chem.*, **173**, 371 (1948).
10. Morgan, K. Z., *J. Phys. and Colloid Chem.*, **51**, 984 (1947).

PERBORATE AS SUBSTRATE IN A NEW ASSAY OF CATALASE[*]

By ROBERT N. FEINSTEIN

(From the Toxicity Laboratory and the Department of Biochemistry, University of Chicago, Chicago)

The comparison of catalase activity in different tissues or animals is time-consuming and frequently of dubious significance. The commonly used (1) temperature of 0–10° for the assay of catalase is less convenient than room temperature or the temperature of 37° usually available in biochemical laboratories. The rapid destruction of catalase by hydrogen peroxide necessitates a series of aliquots, the analyses of which are then used for an extrapolation back to zero time. The formation of oxygen sometimes leads to difficult and inaccurate pipetting of these aliquots. On the other hand, the gasometric determination of catalase (2–6) requires special flasks and is not adapted to large numbers of catalase assays. Usual methods of catalase assay, moreover, in our hands have yielded results which were often very far from linearity with respect to enzyme concentration.

Sodium perborate has been found to serve excellently as substrate for catalase, and an assay method has been devised which, in our hands, avoids most of the difficulties enumerated above.

EXPERIMENTAL

In general, perborate remaining after catalase action has been determined by titration with $KMnO_4$ in the presence of H_2SO_4, exactly as is customary in the determination of hydrogen peroxide. Ordinarily a drop of 1 per cent $MnCl_2$ has been added before titration, to avoid the initial lag in decolorization of $KMnO_4$. The enzyme activity of the incubating mixture is halted by the addition of an equal volume of 2 N H_2SO_4. The acid serves also in the subsequent titration.

Characteristics of Perborate As Substrate—Sodium perborate in aqueous solution is readily attacked by catalase to yield oxygen, with a corresponding reduction in permanganate-reducing ability. Other hyperoxygenated salts, *viz.* potassium persulfate, potassium permanganate, potassium perchlorate, and potassium periodate, did not produce any gas under the influence of catalase.

Tetrahydrated sodium perborate, $NaBO_3 \cdot 4H_2O$, forms a saturated solution at approximately 1.5 per cent at room temperature. The solid salt is relatively stable, but a partial decomposition, if not excessive, is not harmful in the assay, since an adequate excess of $NaBO_3$ is present, and the products of decomposition of $NaBO_3$ have not been observed to exhibit any deleterious effect on the enzyme, although they are stated by Sborgi and Nocentini (7) to affect the spontaneous decomposition of $NaBO_3$. Aqueous solutions of $NaBO_3$ decompose slowly, depending upon the temperature and the pH. It has been customary to include, with each set of catalase assays, a pair of flasks containing perborate and buffer as in the test flasks, but with water replacing the tissue or enzyme source. These controls are then used as base points from which to calculate the perborate destroyed by the enzyme.

Aqueous solutions of $NaBO_3$ are decreased in stability with increase in temperature (7, 8). Under the conditions of routine assay, it has been found that a solution of $NaBO_3$ loses approximately 0.9 per cent of its $KMnO_4$-reducing capacity when maintained at pH 6.8 for 2 hours at room temperature, but loses only 0.3 per cent in the presence of the acid used to halt the enzyme reaction (about pH 0.9).

Destruction of Catalase by Perborate—Like hydrogen peroxide, perborate is destructive to catalase. A mixture of the perborate and catalase solutions (as described below under "Assay procedure") was incubated at 37° for 20 minutes, then divided into two parts. More catalase was added to one part, and more perborate was added to the other. The amounts added were equivalent to those initially present. It was found that in the portion to which more catalase was added further decomposition of the residual perborate took place; in that portion receiving additional perborate, no further decomposition took place.

The destruction of the enzyme by $NaBO_3$ is not as marked at 37° as is the destruction of catalase by hydrogen peroxide, and this is probably the reason for the greater consistency with respect to enzyme concentration when $NaBO_3$ is used as substrate. During a 10 minute incubation, increasing the temperature from 0° to 37° caused a 62 per cent increase in the enzymatic decomposition of $NaBO_3$, but only a 6 per cent increase in decomposition of hydrogen peroxide.

Effect of pH—Fig. 1 indicates the effect of pH on the decomposition of $NaBO_3$ by catalase. There appears to be no sharp pH optimum, but rather a wide band of optimal activity, ranging approximately from pH 7 to 8. In the actual assay, pH 6.8 has been used, since at this point the non-enzymatic decomposition of $NaBO_3$ is slightly lower than at a higher pH, while the enzymatic process is at or near the optimum.

Effect of Buffer—Saturated sodium perborate (about pH 9.9) when

neutralized to pH 6.8 is itself a fairly strong buffer, although pH 6.8 is in the lower part of its buffering range. Although no significant difference in assay result was observed in the presence or absence of phosphate buffer, the pH was observed to rise slightly in the absence of the added buffer. This is unlikely to affect the assay results, since, as noted above, the optimum pH range is a broad band. However, since the phosphate buffer does no harm to the assay, it was considered desirable to retain it and maintain the pH as nearly constant as possible.

Non-Linearity with Time—Presumably because of the destruction of catalase by $NaBO_3$, the enzyme activity continuously decreases with time,
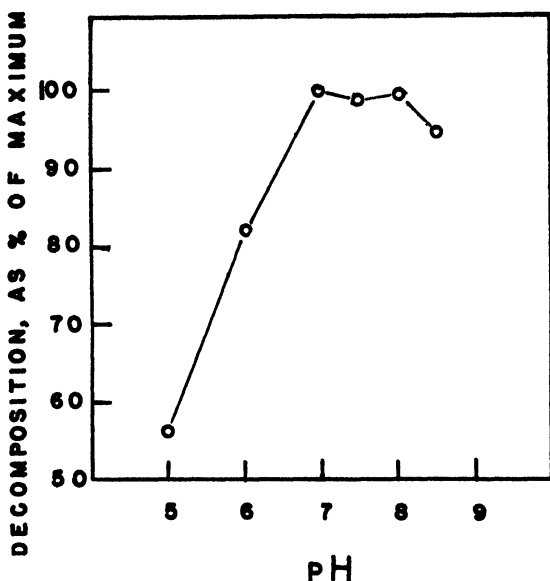
FIG. 1. Effect of pH on decomposition of $NaBO_3$

and it is necessary to select an arbitrary incubation time for the assay. Since an isolated assay requires four flasks, and since 1 minute pipetting intervals are convenient, a 5 minute period has been selected. At the end of the 5 minutes, the reaction rate has already fallen off to approximately 10 per cent of the rate of the 1st minute, but since, as noted below, the percentage decrease is apparently the same regardless of the initial amount of catalase, this seems not to impair the usefulness of the assay method.

Effect of Substrate Concentration—Within certain limits, the total initial amount of perborate present is without effect on the amount of perborate destroyed. The maximum concentration of substrate is limited by the

solubility of the sodium perborate.   The lower limit varies somewhat with the concentration of enzyme and the time of incubation, but in no tissue or set of circumstances tested did a reduction of 20 per cent in the initial amount of substrate affect the assay result.   In the case of previously untested tissues, this simple procedure may be used as a criterion of adequacy of substrate.
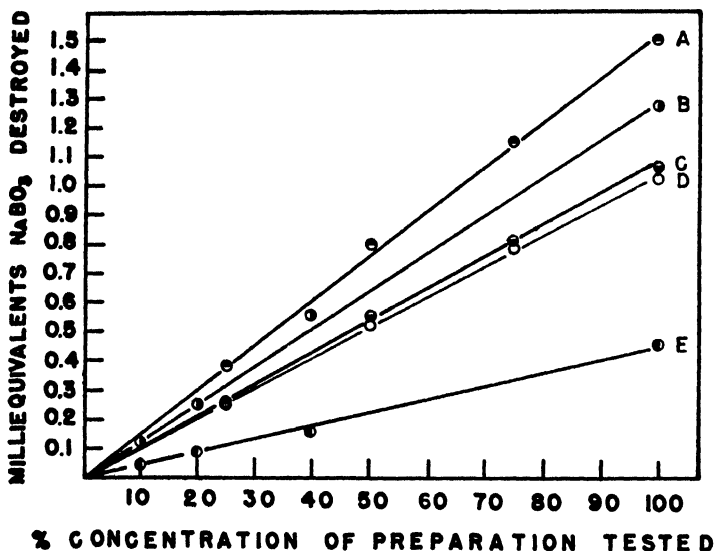
Fig. 2. Proportionality of perborate decomposition to concentration of enzyme source.   Concentrations of enzyme preparations were selected for convenience, and the relative slopes of the various curves are without significance.   The nature and 100 per cent concentration of the various preparations are as follows: Curve A, Armour catalase (technical), 10.0 mg. per ml.; Curve B, rat kidney homogenate, 4.0 mg. per ml.; Curve C, rat liver homogenate, 2.0 mg. per ml.; Curve D, catalase Sarett (Vita-Zyme Laboratories), 2.0 mg. per ml.; Curve E, rat spleen homogenate, 20.0 mg. per ml.   The conditions were those specified under "Assay procedure."

Linearity with Enzyme Concentration—Fig. 2 demonstrates that, under the conditions of this assay method, the amount of $NaBO_3$ decomposed is directly proportional to the concentration of the enzyme, both in crude and semipurified catalase preparations.

Comparison with Kat. f. Assay Method—Table I presents a comparison of assays performed by the conventional method (1) and by the method described in this paper.   Two points in particular will be noted from Table I: (a) the relative activity of the various tissues and preparations, as determined by the two methods, is in approximately the same order, and (b) the perborate pairs are generally more consistent, despite dilu-

tion, than are the *Kat. f.* pairs. In our hands, the determination of the *Kat. f.* of various crude preparations has frequently been accompanied by deviations of several hundred per cent, as indicated, for example, by the assay of Armour catalase shown in Table I. This is less a dilution effect than a simple lack of reproducibility by the *Kat. f.* method.

It should be pointed out in addition that to obtain data for the *Kat. f.* determinations required 3 times the number of pipettings and titrations, over twice the incubation time, and considerably more time for calculations than to obtain data for the $NaBO_3$ method.

TABLE I .

Comparison of Conventional Kat. f. Determination and Perborate Assay Method

Enzyme source	Kat. f. determination		Perborate assay	
	Dilutions used	Kat. f.*	Dilutions used	Activity*
Catalase Sarett (Vita-Zyme Laboratories)	1:1000	93	1:1000	0.994
	1:2000	106	1:2000	1.008
Rat liver homogenate	1:500	94	1:500	0.814
	1:1000	127	1:1000	0.810
" kidney homogenate	1:250	50	1:250	0.331
	1:500	38	1:500	0.303
Armour catalase (technical)	1:1000	21	1:200	0.273
	1:5000	125	1:1000	0.258
Rat lung homogenate	1:50	3.9	1:50	0.053
	1:100	4.2	1:100	0.055
" spleen homogenate	1:50	5.0	1:50	0.051
	1:100	4.3	1:100	0.048
" brain homogenate	1:10	0.34	1:10	0.0074
	1:20	0.73	1:20	0.0072

* Perborate assay activity is expressed as milliequivalents of $NaBO_3$ destroyed per mg. of wet weight of enzyme source under conditions specified in the text. *Kat. f.* values are here expressed as the monomolecular K values, extrapolated to zero time and divided by the gm. of wet tissue.

Assay Procedure—The substrate is 1.5 per cent $NaBO_3 \cdot 4H_2O$, filtered if necessary, and adjusted to pH 6.8 with concentrated HCl. 8 ml. of substrate and 1.5 ml. of M/15 phosphate buffer, pH 6.8, are placed in a series of four 125 ml. Erlenmeyer flasks. The flasks are weighted with large metal washers, covered with small watch-glasses, and immersed in a bath at 37°. After 15 to 20 minutes 0.5 ml. of H_2O is added to the first flask, 0.5 ml. of enzyme source (tissue homogenate, etc.), ordinarily in two different concentrations, to the next two flasks, and 0.5 ml. of H_2O to a fourth flask. After exactly 5 minutes, 10 ml. of 2 N H_2SO_4 are added, and the flasks are then titrated with standard $KMnO_4$, about 0.05 N. In the case of many tissues, the necessary dilution is so great that

the KMnO₄-reducing property of the enzyme source is negligible. In the case of those tissues with weak catalase activity (*e.g.* brain, testis), sufficiently concentrated homogenates are required to necessitate the determination of the KMnO₄-reducing property of the tissue. To determine this, an additional flask may be used, containing tissue, buffer, and water instead of perborate. It has not been found necessary, under any circumstance, to filter the incubated mixtures.

DISCUSSION

Catalase is considered to be a highly specific enzyme (1). It is doubtful that the action of catalase on $NaBO_3$ refutes this specificity, since it is well known (9–12) that perborate in aqueous solution produces some hydrogen peroxide, and it is probable that this serves as the actual substrate. It is, in fact, possible that this is the reason for the more successful use of $NaBO_3$ as substrate, since Morgulis (13) has demonstrated that the inactivation of catalase by its substrate is dependent on the concentration of the hydrogen peroxide; the aqueous $NaBO_3$ probably provides a constant low concentration of peroxide, which is then acted upon by the catalase. At no time has any evidence been obtained that the catalase activity is limited by the production of hydrogen peroxide from $NaBO_3$.

SUMMARY

Sodium perborate is decomposed by catalase. This has been made the basis of a method of catalase assay which is rapid and convenient, and which yields results that are proportional to the concentration of the enzyme.

BIBLIOGRAPHY

1. Sumner, J. B., and Somers, G. F., Chemistry and methods of enzymes, New York (1947).
2. Bodansky, M., *J. Biol. Chem.*, **40**, 127 (1919).
3. Harvey, R. B., *J. Gen. Physiol.*, **2**, 253 (1920).
4. Morgulis, S., *J. Biol. Chem.*, **47**, 341 (1921).
5. Fujita, A., and Kodama, T., *Biochem. Z.*, **232**, 15, 20 (1931).
6. Thompson, R. R., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 585 (1942).
7. Sborgi, U., and Nocentini, G., *Gazz. chim. ital.*, **51**, pt. 1, 289 (1921); *Chem. Abstr.*, **16**, 9 (1922).
8. Melikoff, P. G., and Pissarjewsky, L., *Ber. chem. Ges.*, **30**, 3144 (1897); **31**, 678, 953 (1898); **32**, 341 (1899); from Mellor, J. W., A comprehensive treatise on inorganic and theoretical chemistry, London (1924).
9. Pissarjewsky, L., *Z. anorg. Chem.*, **32**, 341 (1902).
10. Pissarjewsky, L., *Z. phys. Chem.*, **43**, 160 (1903).
11. Mathews, J. M., *J. Ind. and Eng. Chem.*, **3**, 191 (1911).
12. Lenz, W., and Richter, E., *Z. anal. Chem.*, **50**, 537 (1911).
13. Morgulis, S., *J. Biol. Chem.*, **92**, 377 (1931).

STUDIES ON RIBONUCLEIC ACID*

I. PREPARATION FROM PANCREAS

By STANLEY E. KERR and KRIKOR SERAIDARIAN

(From the Department of Biochemistry, American University of Beirut, Beirut, Lebanon)

In spite of studies extending over some 50 years, beginning with Hammarsten's preparation of the so called β-nucleoprotein of pancreas (1), there seems to be no agreement among investigators as to the composition of the ribonucleic acid (RNA) of animal tissue. Having in mind the possibility that various procedures for the isolation of nucleic acid may yield different fractions of mixed material actually present in the cell, we have first given our attention to the improvement of methods of preparation, maximum yield being a chief objective. The procedure described below is a step in this direction, yielding 15 to 20 per cent of the RNA in the pancreas without subjecting the compound to any drastic treatment.

EXPERIMENTAL

The entire procedure is carried out in the cold room. Beef pancreas,[1] packed in ice at the slaughter-house, is freed from connective tissue and fat, ground in an ordinary meat chopper, then weighed and stirred mechanically with 1½ volumes of cold 0.14 M NaCl for an hour. The mixture is centrifuged for 30 minutes at the maximum speed permissible for the bottles used, and the residue is extracted in the same way a second time. The combined extracts are brought to pH 4.2 (glass electrode) by addition of about 0.02 volume of N HCl.

The acid precipitate, which contains all of the nucleoprotein in the extracts, is centrifuged for 30 minutes at maximum speed and the supernatant is discarded. The precipitate is removed from the centrifuge bottles by shaking with 0.14 M NaCl. The total volume of NaCl used should not exceed 800 cc. per kilo of pancreas, about half of it being used for the first shaking. While being stirred mechanically, the mixture is brought to pH 6.8 to 7.0 by the slow addition of N NaOH (about 25 cc. per kilo of pancreas). It is important that at no time should the mixture become alkaline. Crystalline NaCl (18 gm. per 100 cc.) is added

[1] It is convenient to work up a batch of pancreas equal to 0.4 the capacity of the centrifuge available. Thus for a centrifuge carrying four 300 cc. cups, 480 gm. of tissue may be handled at a time.

to make a half saturated (3 M) solution, and stirring is continued until the salt is dissolved. The mixture is allowed to stand at least 36 hours with occasional stirring to permit dissociation of the nucleic acid from the protein. The mixture of salt and insoluble residue is adjusted to pH 4.2 with N HCl (about 25 cc. per kilo of pancreas) and the precipitated protein is removed by filtration through large fluted filters with the help of a filter aid (Hyflo Super-Cel). Filtration overnight was required.

To the salt filtrate containing the nucleinate 3 volumes of 95 per cent ethyl alcohol are added with stirring. The acid sodium nucleinate which precipitates is separated by centrifugation, then suspended in water (500 cc. per kilo of pancreas are used) and dissolved by the careful addition of N NaOH, alkaline reaction always being avoided. The solution is again brought to pH 4.2 by addition of 0.2 N HCl, in order to precipitate contaminating protein or nucleoprotein.[2]

The supernatant is treated with 3 volumes of alcohol to precipitate the nucleinate. At this point the recovery is about 25 per cent of the RNA in the original pancreas, but the product still contains a trace of protein, some of which is not precipitated at pH 4.2 but remains in solution when the RNA is precipitated by alcohol. By redissolving the acid nucleinate and reprecipitating with alcohol,[3] a product is obtained which no longer responds to the biuret test. It is washed twice with 95 per cent alcohol and once with ether, and then dried over H_2SO_4 *in vacuo*.[4] The yield varies from 15 to 20 per cent of the RNA in the pancreas.

The acid sodium nucleinate obtained by this procedure is a pure white salt, easily soluble in water, forming a clear non-viscous solution. The diphenylamine (2) and cystine-H_2SO_4 (3) reactions for DNA are negative unless samples of at least 20 mg. are tested. Quantitative determinations by these methods indicate that the preparations may contain from 1 to 1.9 per cent of DNA, if these reactions are completely specific in the presence of such large excesses of RNA.

Whether the RNA prepared by our procedure is a single compound or a mixture of different ribonucleic acids is uncertain. It is completely precipitated by MacFadyen's uranyltrichloroacetic acid reagent as modified by Zittle (4). Attempts were made to fractionate it by the procedures

---

[2] Because of its high concentration some nucleic acid will be carried down with the protein precipitate. We generally discard this but, if desired, it may be recovered by dissolving the precipitate at pH 7 and reprecipitating at 4.2, the supernatant being added to the main batch of nucleic acid.

[3] Occasionally, when the preparation is nearly pure, alcohol fails to precipitate the RNA. Addition of 1 or 2 cc. of saturated NaCl solution corrects this difficulty, causing flocculation of the precipitate.

[4] In case further fractionation by the procedures of Levene and Jorpes (5) or of Chantrenne (6) is desired, the alcohol precipitate should not be dried.

of Levene and Jorpes (5) and Chantrenne (6). The latter has shown that precipitation with 50 per cent acetic acid alters yeast nucleic acid, since on reprecipitation with the same reagent a new fraction of the precipitate becomes soluble. By precipitating yeast RNA from solution with an equal volume of an acetone-acetic acid mixture (20 per cent acetone and 40 per cent acetic acid) Chantrenne secured a fraction which on reprecipitation was found to be entirely insoluble. On attempting to fractionate pancreas RNA with Chantrenne's reagent we obtained colloidal solutions. In a personal communication Chantrenne states that nucleic acid prepared from a Danish yeast also gave colloidal solutions with the acetone-acetic acid reagent, but this difficulty was avoided by increasing the acetone content of the precipitant to 40 per cent. We find, however, that this modified reagent also gives colloidal solutions with pancreas RNA.

A precipitant composed of 20 parts of acetone, 40 of glacial acetic acid, and 40 of 0.14 M NaCl, added to an equal volume of pancreas nucleic acid solution, gives a flocculent precipitate containing 78 to 86 per cent of the nucleic acid. On redissolving the precipitate in water and again adding the reagent only 50 per cent of the nucleic acid precipitates. Pancreas RNA is obviously decomposed by the acetone-acetic acid reagent, as well as by the acetic acid reagent of Levene and Jorpes (5).

Approximately 15 per cent of our RNA preparations is a low polymer fraction, since 5 volumes of acetic acid precipitate 83.7 per cent of the RNA (based on phosphate determinations), the Chantrenne NaCl reagent precipitates 78.8 per cent, and on dialysis in the cold room 16.5 per cent of the material is found in the dialysate after 96 hours.

The so called high polymer fraction prepared by any one of the three procedures just mentioned appears to be unstable in aqueous solution at room temperature. In an effort to find a procedure for preparing a stable high polymer fraction, a single specimen of freshly prepared undried pancreas RNA was subjected to the several manipulations noted below. The distribution of the material on dialysis or precipitation is indicated in terms of per cent of total phosphate of the sample immediately preceding.

In the cold room 18.5 per cent dialyzed out in 106 hours, the loss diminishing to negligible amounts in this period. Of the remaining non-dialyzable RNA 28.6 per cent became dialyzable on continuation of the procedure at room temperature for another 106 hours, the loss again diminishing to a negligible quantity. Of the material finally left in the cellophane sac only 56.3 per cent was precipitated by the Chantrenne NaCl reagent.

The Chantrenne NaCl reagent precipitated 78.8 per cent. The precipitate was redissolved and dialyzed, 25.6 per cent being lost in 60 hours.

The non-dialyzable portion was precipitated with 3 volumes of alcohol and the precipitate redissolved and again dialyzed, 33.8 per cent being lost in 48 hours.

5 volumes of glacial acetic acid precipitated 83.7 per cent. The precipitated fraction was dissolved in water and dialyzed, 27.8 per cent being lost. The non-dialyzable portion was precipitated with 3 volumes of alcohol and the precipitate redissolved and dialyzed, 32.8 per cent being lost in 48 hours.

The unfractionated RNA was precipitated with 3 volumes of alcohol, dehydrated with alcohol and ether, and dried *in vacuo*, one-half at 100° and the other at 20°. Both specimens were then dissolved and dialyzed. The specimen dried at 20° lost 11 per cent to the dialysate in 30 hours, and from the remaining non-dialyzable material (which was dried) the Chantrenne reagent precipitated only 26.2 per cent. The specimen dried at 100° lost 18.5 per cent on dialysis, the Chantrenne reagent precipitating 26.2 per cent of the dried residue.

From the above it is evident that none of these procedures is suitable for the preparation of a high polymer fraction in the dry state. A possible solution of this difficulty may be found by drying in the frozen state. Meanwhile it seems advisable that preparations be kept in the cold room as finally precipitated by alcohol without drying, if undenatured material is desired.

DISCUSSION

In the preparation of ribonucleic acid avoidance of contamination with desoxyribonucleic acid (DNA) has offered difficulties. Levene and Jorpes (5) state that the proportion of DNA may be lowered to less than 1 per cent by precipitation with 10 volumes of glacial acetic acid. In our hands this procedure yielded a product containing more than 3 per cent DNA. Further, it has been shown by Chantrenne (6) that this method of purification causes a change in the character of yeast RNA. Mirsky and Pollister (7), taking advantage of the fact that ribonucleoprotein (RNP) but not desoxyribonucleoprotein (DNP) dissolves readily in 0.14 M NaCl, prepared the former from thymus by a procedure similar in principle to ours, which we had developed independently.[5] Although DNP is known to be insoluble in 0.14 M NaCl (7, 9) we regularly found our first preparations of RNA from pancreas to contain from 5 to 6 per cent of DNA and finally traced the contamination to the use of a high speed

---

[5] Credit belongs to Bensley (8) for the means of purification of DNP later used by Mirsky and Pollister (7). Although Bensley did not recognize the true nature of the "plasmosin" which he had isolated, he described clearly its solubility in strong salt solution and its precipitation on dilution.

mixer for mincing the pancreas. This, we believe, liberates a quantity of nuclei and some chromatin threads which remain suspended when the extracted tissue is separated by centrifugation, and are consequently included with the RNP when this is precipitated at pH 4.2. Mirsky and Pollister (7) removed DNP by washing the precipitate with 0.5 M NaCl and water, and by two reprecipitations. This time-consuming procedure may be avoided if the tissue is merely ground in a meat chopper, in which case no DNP is found in the saline extracts. Under the same conditions extracts prepared from tissue minced in a Waring blendor contained 25 per cent of the DNA in the pancreas. An additional reason for avoiding reprecipitation of the nucleoprotein is the loss of RNA which results. When the nucleoprotein is dissolved at neutrality and reprecipitated at pH 4.2, the acid supernatant is found to contain a considerable amount of the RNA. During this process part of the protein is detached from the nucleic acid, as shown by the ratio of $N:P$ in the material precipitated by alcohol.

In the preparation of protein-free nucleic acid, separation of the protein component offered the greatest difficulty in a procedure otherwise very simple. Feulgen accomplished this (10) by digesting the protein with trypsin, and by precipitating the RNA with crystal violet. Hammarsten (11) obtained protein-free RNA by repeated precipitation with $CaCl_2$. Jorpes (12) denatured the protein in the crude nucleoprotein precipitate by means of alcohol, then separated whatever protein remained by precipitation of the barium salt of RNA from a saline extract at pH 6.8. Each of these procedures results in considerable loss of RNA. The ease with which RNA separates from its protein when the nucleoprotein is dissolved and reprecipitated by acid suggested to us that the dissociation might be accomplished more rapidly and completely in the presence of strong NaCl solution, as was done by Hammarsten in the preparation of protein-free DNA (13). The use of saturated NaCl is inconvenient, since it separates together with the RNA in the subsequent precipitation by alcohol. Half saturated NaCl, however, is not precipitated, and permits the dissociation of the protein with equal ease. The process is nearly complete within 6 hours, but protein-free RNA is obtained with fewer reprecipitations when the digestion with NaCl is continued for 36 to 48 hours. We were unable to remove the protein completely from pancreas RNP by the procedure of Sevag et al. (14). After shaking at pH 6.8 with a chloroform-amyl alcohol mixture twenty-one times for 20 minutes each, the preparation still contained protein.

It appears that the RNA prepared from pancreas by the procedure described above differs from that of yeast in its stability. From yeast RNA a stable high polymer fraction may be secured by precipitation with

an acetone-acetic acid reagent (6), whereas the corresponding fraction prepared from pancreas RNA seems to be depolymerized by this treatment. Further, the fraction of pancreas RNA found to be non-dialyzable in the cold room proceeds to lose a further fraction when dialysis is continued at room temperature. Further study of these changes and also those caused by ribonuclease will be reported later.

The composition of pancreas RNA and methods of analysis, including determination of the purines and pyrimidine nucleotides, will be discussed in the following papers of this series.

SUMMARY

A simple procedure is described for the preparation of ribonucleic acid from beef pancreas with 20 per cent yield. The method involves extraction of the tissue with 0.14 M NaCl, precipitation of the nucleoprotein at pH 4.2, dissociation of protein from nucleic acid by means of 3 M NaCl, removal of protein at pH 4.2, and precipitation of nucleinate by alcohol.

Attempts to fractionate the pancreas ribonucleic acid by precipitation with 83 per cent acetic acid or with Chantrenne's acetone-acetic acid reagent resulted in preparations less completely precipitated by the same reagents and more readily diffusible.

About 15 per cent of the material dialyzes through cellophane in the cold room, but further dialysis at room temperature results in additional loss of material, the non-diffusible fraction presumably being depolymerized.

BIBLIOGRAPHY

1. Hammarsten, O., *Z. physiol. Chem.*, **19**, 19 (1894).
2. Sevag, M. G., Smolens, J., and Lackman, D. B., *J. Biol. Chem.*, **134**, 523 (1940).
3. Stumpf, P. K., *J. Biol. Chem.*, **169**, 367 (1947).
4. Zittle, C. A., *J. Biol. Chem.*, **163**, 119 (1946).
5. Levene, P. A., and Jorpes, E., *J. Biol. Chem.*, **86**, 389 (1930).
6. Chantrenne, H., *Bull. Soc. chim. Belges*, **55**, 5 (1946).
7. Mirsky, A. E., and Pollister, A. W., *J. Gen. Physiol.*, **30**, 101 (1946).
8. Bensley, R. R., *Anat. Rec.*, **72**, 351 (1938).
9. Mirsky, A. E., and Pollister, A. W., *Proc. Nat. Acad. Sc.*, **28**, 344 (1942).
10. Feulgen, R., *Z. physiol. Chem.*, **108**, 147 (1919–20).
11. Hammarsten, E., *J. Biol. Chem.*, **43**, 243 (1920).
12. Jorpes, E., *Acta med. Scand.*, **68**, 503 (1928).
13. Hammarsten, E., *Biochem. Z.*, **144**, 383 (1924).
14. Sevag, M. G., Lackman, D. B., and Smolens, J., *J. Biol. Chem.*, **124**, 425 (1938).

THE SPECIFICITY OF LEUCINE AMINOPEPTIDASE

II. OPTICAL AND SIDE CHAIN SPECIFICITY*

By EMIL L. SMITH and W. J. POLGLASE

(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Biological Chemistry and Medicine, University of Utah College of Medicine, Salt Lake City)

The enzyme, leucine aminopeptidase, which was first identified by Linderstrøm-Lang (3), has been assumed to be specific for the hydrolysis of peptide bonds involving the leucine carbonyl. This conclusion was derived from the observations that enzymes which hydrolyze glycine and alanine peptides are less stable than the leucine peptidase (3) and may be readily separated by simple purification procedures (4). Since leucinamide, leucylglycine, and leucylglycylglycine are hydrolyzed at about the same rate, and since the acylated derivatives are resistant to hydrolysis, the enzyme was identified as an aminopeptidase which required for its action the presence of a free amino group in the substrate (4). It was later shown that the free amino group did not have to be adjacent to the sensitive peptide bond, since the terminal amide bond of glycyl-L-leucinamide was split by this enzyme (2).

In this investigation we have utilized a number of substrates in studying the rôle of various factors which determine the specificity of this enzyme. The simplest action manifested by leucine aminopeptidase is its hydrolysis of L-leucinamide. It has now been found that the enzyme can split other aliphatic amino acid amides. This may be represented as a modification of R′ in the substrate structure shown where the dotted line indicates the hydrolytic action.

$$R'—\underset{\underset{NH_2}{|}}{CH}—\underset{\underset{O}{\|}}{C}—\vdots—NHR''$$
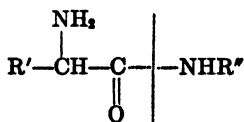
Previous studies indicated that changes in R″ appear to have little effect on the action of this enzyme, since similar rates are obtained with the amide or with dipeptides and tripeptides. Indeed, such a compound as L-leucyl-β-alanine is hydrolyzed at about the same rate as the other

---

substrates (5). We have tested the optical specificity towards this position by utilizing the diastereoisomers, L-leucyl-L-alanine and L-leucyl-D-alanine.

The second type of action shown by this enzyme has also been studied in greater detail, with compounds of the general structure shown where R′ is the isobutyl residue of L-leucine.

$$R'''\!\!-\!\!\underset{\underset{}{}}{\overset{\overset{NH_2}{|}}{C}}H\!\!-\!\!\underset{\underset{O}{\|}}{C}\!\!-\!\!NH\!\!-\!\!\underset{\underset{R'}{|}}{C}\!\!-\!\!\underset{\underset{O}{\|}}{C}\!\!-\!\!NH_2$$
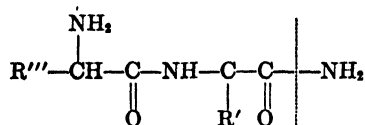
Since glycyl-L-leucinamide is hydrolyzed, the following compounds, L-alanyl-L-leucinamide, D-alanyl-L-leucinamide, and β-alanyl-L-leucinamide, were studied. In this way, further evidence was obtained concerning the essentiality and rôle of the free amino group in the action of the enzyme.

<center>EXPERIMENTAL</center>

Enzyme Preparations—The source of the preparations used in this investigation was hog intestinal mucosa. The fresh washed tissue was ground with sand and extracted with several volumes of cold water. The extract was then clarified at 2° in a Sharples centrifuge and dried from the frozen state.[1] The dried powder is an exceedingly stable source of many intestinal peptidases and may be kept in the cold for some months with no significant loss of activity. For many experiments, the dried powder was extracted with 5 to 10 volumes of distilled water and centrifuged. This is Preparation A.

For the purification of leucine aminopeptidase, the dried powder was suspended at room temperature in 10 volumes of acetone and extracted with occasional stirring for several hours. The suspended material was then collected on a Büchner funnel, washed several times with acetone, and air-dried. About 35 per cent of the weight of the powder was removed by this procedure. The acetone-dried powder was then extracted with about 8 to 10 volumes of water for several hours. Solid ammonium sulfate was added to produce 40 per cent saturation and the precipitate was removed by filtration through fluted filters at 2°. To the filtrate, ammonium sulfate was added to produce first 60 per cent (Preparation B) and then 80 per cent (Preparation C) saturation. These two fractions were dialyzed separately at 2° against distilled water until free of sulfate. The inactive precipitates were removed and the solutions adjusted with 0.1 M NaOH to pH 7.5 to 8.0. Preparations B and C may be stored under toluene at 2° for some months with little change in activity.

Previous preparations of this enzyme made by a similar method from fresh mucosa without lyophylizing gave the highest activity in the 40 to 60 per cent fraction (4). In the present instance, it was found that Preparation B gave the greater yield of enzyme but a higher state of purity was obtained with Preparation C.

Procedure—The enzyme preparations were incubated with 0.01 to 0.04 M MnSO$_4$ for about 3 hours at 40° in the presence of veronal buffer at pH 7.8 to 8.0 in order to achieve the maximal activities (4). This solution was then added to the buffered substrate solution (0.04 M veronal) for subsequent measurement of the hydrolytic reactions. The Mn^{++} concentration in the test solutions was 0.001 to 0.003 M with the exception of the experiments in Table III, where no Mn^{++} was added. The enzyme concentrations given in Tables I to VI are for the final reaction mixtures. The substrate solutions were used at 0.05 M, except for the racemic mixtures, which were at 0.1 M.

Hydrolysis was determined by the measurement of liberated carboxyl groups (6). The proteolytic coefficients (C) were calculated in the usual manner from $C = K/E$, where K is the first order velocity constant expressed in decimal logarithms and E is the enzyme concentration given in mg. of protein N per cc. of test solution. The hydrolysis is given as 100 per cent for the complete splitting of one peptide bond.

Substrates—The following compounds were prepared as previously described: L-leucinamide (2), L-alaninamide (5), glycinamide (7), L-alanyl-glycine (8), and glycyl-L-leucine (9). D-Leucylglycine and D-leucyl-L-tyrosine were commercial preparations obtained from Hoffmann-La Roche, Inc. The synthesis of the following compounds will be presented elsewhere (10): L-leucyl-L-alanine, L-leucyl-D-alanine, L-alanyl-L-leucinamide, D-alanyl-L-leucinamide, β-alanyl-L-leucinamide, L-leucyl-L-alaninamide, and L-alanyl-L-leucine.

DL-*Norleucinamide Hydrochloride*—This was prepared by the direct amidation of 12.7 gm. of DL-norleucine methyl ester hydrochloride (m.p. 122–123°) in 120 cc. of saturated anhydrous methanol-ammonia at 0°. After 40 hours in a pressure bottle at room temperature, the solution was concentrated *in vacuo;* the product crystallized on addition of ether. Yield, 8.0 gm. The compound was recrystallized from methanol-ethyl acetate; m.p. 234–235° with decomposition.

C$_6$H$_{13}$ON$_2$Cl (166.7).   Calculated, N 16.8; found, N 16.9

DL-*Norvalinamide Hydrochloride*—This was prepared from 7.0 gm. of the methyl ester hydrochloride (m.p. 116–117°) as described above. Yield, 4.7 gm; m.p. 240° with decomposition.

C$_5$H$_{13}$ON$_2$Cl (152.6).   Calculated, N 18.4; found, N 18.4, 18.3

DL-α-*Amino-n-butyramide Hydrochloride*—This was prepared from 6.0 gm. of the methyl ester hydrochloride (m.p. 150–151°) as described above. Yield, 4.3 gm; m.p. 222–223°.

$C_4H_{11}ON_2Cl$ (138.6). Calculated, N 20.2; found, N 20.4

Carbobenzoxy-L-*tryptophanamide*—10 gm. of carbobenzoxy-L-tryptophan (11) were dissolved in 125 cc. of methanol and esterified three times at 0° with anhydrous HCl after concentrating repeatedly to dryness each time with methanol. The syrupy ester was then allowed to stand at room temperature for 40 hours in methanol previously saturated with ammonia at 0°. On repeated concentration with methanol, the compound crystallized and was collected with ether. Yield, 7.9 gm. After recrystallization from absolute ethanol, the melting point was 187–188°.

$C_{19}H_{19}O_3N_3$.   Calculated.   C 67.6, H 5.7, N 12.4
337.4   Found.   " 67.1, " 5.6, " 12.2

L-*Tryptophanamide Hydrochloride*—This was prepared by reduction of 1.0 gm. of the carbobenzoxy derivative in 30 cc. of methanol with 0.3 cc. of concentrated HCl and a palladium catalyst. After 1 hour, the catalyst was filtered off, the pink filtrate was concentrated to about 10 cc., and ether was added. The precipitate was dissolved in 50 cc. of 70 per cent ethanol and decolorized with norit A. The filtered solution was concentrated to 4 cc. and ethanol and ether were added. The compound crystallized in rectangular plates. Yield, 0.36 gm.; m.p., 257–260° with decomposition.

$$[\alpha]_D^{25} = +24° \text{ (1.6\% in water)}$$

This compound was also prepared by direct amidation of 5.0 gm. of L-tryptophan methyl ester hydrochloride as described above for DL-norleucinamide hydrochloride. After removal of the methanol-ammonia by repeated concentration *in vacuo*, the compound crystallized from methanol-ether. Yield, 3.25 gm. It was recrystallized from methanol-ether.

$C_{11}H_{14}ON_3Cl$.   Calculated.   C 55.1, H 5.9, N 17.5
239.7   Found.   " 55.0, " 6.0, " 17.4
$$[\alpha]_D^{25} = +24° \text{ (1.1\% in water)}$$

D-*Leucinamide Acetate*—We are indebted to Dr. William H. Stein of the Rockefeller Institute for Medical Research for this compound and the description of its properties. This substance was prepared by the same procedure as described for the analogous L compound (12); m.p., 128–129°.

$C_8H_{18}O_3N_2$.   Calculated.   C 50.5, H 9.5, N 14.7
190.2   Found.   " 50.4, " 9.5, " 14.6
$$[\alpha]_D^{24} = -9.2° \text{ (5\% in water)}$$

Behrens and Bergmann (12) reported +9.25° for L-leucinamide acetate. Smith and Slonim (2) found +9.5° for the hydrochloride.

Results

Action on Amino Acid Amides—Table I shows the results for the action of Preparations B and C on certain amino acid amides. In each instance the hydrolysis followed the kinetics of a first order reaction within the precision of the measurements (±5 per cent) to at least 70 to 80 per cent of completion. For the racemic compounds, no evidence was obtained for

TABLE I

Hydrolysis of Amino Acid Amides by Leucine Aminopeptidase

Each proteolytic coefficient (*C*) represents the average of two independent runs.

Substrate	Preparation B			Preparation C		
	Enzyme concentration	*C*	Relative activity	Enzyme concentration	*C*	Relative activity
	mg. protein N per cc.		*per cent*	*mg. protein N per cc.*		*per cent*
L-Leucinamide..........	0.0117	0.18	100	0.00072	3.1	100
DL-Norleucinamide......	0.0117	0.18	100	0.00072	3.2	103
DL-Norvalinamide.......	0.0117	0.14	78	0.00072	2.5	81
DL-α-Amino-n-butyramide	0.0235	0.071	40	0.00072	1.5	48
L-Alaninamide..........	0.353	0.011	6.1	0.0086	0.20	6.5
Glycinamide............	0.353	0.00064	0.35	0.0086	0.010	0.32
L-Tryptophanamide.....	0.117	0.014	7.8	0.0029	0.80	26

the splitting of the D-amino acid amide. Moreover, tests with D-leucinamide, D-leucylglycine, and D-leucyl-L-tyrosine showed that these compounds are completely resistant to the action of the aminopeptidase, even at high enzyme concentrations. It was also found that the presence of an equimolar amount of D-leucinamide had no effect on the rate of hydrolysis of the L isomer. Therefore, it appears that the rates of hydrolysis observed for the racemic mixtures reflect the true rates for the L compounds alone.

Although Preparation C has a specific activity about seventeen times greater than that of Preparation B, the relative rates for all of the aliphatic compounds are the same within the precision of the measurements. However, with L-tryptophanamide the relative rates for the two preparations are significantly different. While it cannot be decided from the present evidence whether leucine aminopeptidase possesses any action on the aromatic compound, there can be little doubt that there is present in these preparations a distinct enzyme which hydrolyzes L-tryptophanamide.

The data in Table I suggest that the various aliphatic amides are all hydrolyzed by the same enzyme. This assumption has been tested further by the use of the crude Preparation A. Some experimental difficulties were experienced in using Preparation A with the compounds that are slowly hydrolyzed, since the crude preparation shows some autolysis during the time required for significant hydrolysis to occur. Satisfactory data could not be obtained for the splitting of glycinamide; the results for the other compounds are given in Table II. It is apparent that, while the data are not too precise, the extent of purification (over 200-fold) is about the same for all of the compounds.

Since it is well known that leucine aminopeptidase is strongly activated by Mn^{++} (4, 13), a further test of the identity of the various actions can

TABLE II

Hydrolysis of Amino Acid Amides by Preparation A

Substrate	Enzyme concentration	C	Activity in relation to Preparation C
	mg. protein N per cc		*per cent*
L-Leucinamide ...	0.114	0.015	0.5
DL-Norleucinamide	0.114	0.010	0.3
DL-Norvalinamide	0.284	0.012	0.5
DL-α-Amino-n-butyramide	0.284	0.0057	0.4
L-Alaninamide	0.568	0.0016	0.8

be made by determining the relative extent of activation for the different amides. The rates of hydrolysis of glycinamide and L-alaninamide in the absence of added Mn^{++} were too slow to be reliable. The proteolytic coefficients for the other compounds are shown in Table III, together with the percentage of activity as compared to the data for the fully activated preparations given in Table I. It is apparent that the rates of hydrolysis of all of the amides are increased by the addition of Mn^{++}, and that in each case the activity is about 15 per cent of that given by the fully activated preparations.

Thus, the present evidence is in accord with the assumption that all of the aliphatic amino acid amides are hydrolyzed by the same enzyme, since the relative activities are about the same for preparations at three different states of purity, and since the hydrolysis of these compounds is influenced in the same manner and to the same relative extent by the addition of Mn^{++}.

Optical Specificity of Peptide Hydrolysis—Leucine aminopeptidase shows an absolute optical specificity towards amides or peptides, since the

residue bearing the free amino group must be of the L configuration. These data are shown in Table IV. Previous results had also shown that D-leucylglycine is resistant to the aminopeptidase (4). Table IV also shows the results obtained when the optical configuration of the residue possessing the peptide nitrogen has been altered. Comparison of the rates of L-leucyl-L-alanine and L-leucinamide shows that the two compounds are hydrolyzed at about the same velocity. This was not unexpected, since L-leucylglycine is also hydrolyzed at the same rate as the amide. However, L-leucyl-L-alanine is hydrolyzed by both preparations about twenty-three times more rapidly than its diastereoisomer, L-leucyl-D-alanine. It had previously been found (8) that a crude glycerol extract

TABLE III

Hydrolysis of Amino Acid Amides without Added Mn^{++}

The amount of activity has been calculated in comparison with the fully activated preparations described in Table I.

Substrate	Preparation B		Preparation C	
	C	Activity compared to fully active enzyme	C	Activity compared to fully active enzyme
		per cent		*per cent*
L-Leucinamide............	0.027	15	0.44	14
DL-Norleucinamide............	0.027	15	0.55	17
DL-Norvalinamide.............	0.021	15	0.25	10
DL-α-Amino-n-butyramide.... .	0.0071	10	0.13	9

of intestinal mucosa splits both of these compounds, but quantitative comparisons had not been made.

Specificity of Action on Dipeptide Amides and Dipeptides—It has been noted that leucine aminopeptidase hydrolyzes glycyl-L-leucinamide at the terminal peptide linkage (2). It has now been observed that both bonds of L-alanyl-L-leucinamide are rapidly hydrolyzed. Table V shows that with Preparation C, at an enzyme concentration of 0.00064 mg. of protein N per cc., complete hydrolysis occurs. At a lower enzyme concentration (0.00029 mg. of protein N per cc. of Preparation C and with Preparation B), the initial splitting is apparently first order and these rates may be compared with those of the other substrates. It was also found that the hydrolysis of L-alanyl-L-leucine by Preparation C is more rapid than that of any other known peptide or amide, since C for the peptide is 6.2 while, for L-leucinamide, C is 3.1. It is likely that the initial hydrolysis of the dipeptide amide occurs at the peptide linkage adjacent to the free amino group. The proteolytic coefficients given for the compounds with

TABLE IV

Optical Specificity of Peptide and Amide Hydrolysis

Substrate	Preparation B		Preparation C	
	Enzyme concentration	*C*	Enzyme concentration	*C*
	mg. protein N per cc.		*mg. protein N per cc.*	
L-Leucinamide............	0.0112	0.18	0.00072	3.1
D-Leucinamide..........			0.0086	<0.001
D-Leucyl-L-tyrosine...........			0.0086	<0.001
D-Leucylglycine...............			0.0086	<0.001
L-Leucyl-L-alanine.............	0.0117	0.20	0.00072	3.1
L-Leucyl-D-alanine.....	0.353	0.0095	0.0086	0.13

TABLE V

Hydrolysis of Dipeptide Amides and Dipeptides

Substrate	Preparation B				Preparation C			
	Enzyme concentration	Time	Hydrolysis	*C*	Enzyme concentration	Time	Hydrolysis	*C*
	mg. protein N per cc.	*hrs.*	*per cent*		*mg. protein N per cc.*	*hrs.*	*per cent*	
L-Alanyl-L-leucinamide	0.0059	0.5	17	0.45	0.00029			8.1
		1	33	0.49	0.00064	0.5	38	10
		1.5	44	0.48		1	65	12
		2	51	0.44		1.5	90	17
		17	167			2	107	
						2.25	115	
						5	171	
						19	204	
D-Alanyl-L-leucinamide	0.053	18	36	0.00050	0.0086	18	19	0.010
		24	43	0.00048		24	26	0.010
		48	74	0.00058		48	46	0.011
β-Alanyl-L-leucinamide	0.353	18	10	0.00012	0.0086	48	13	0.0024
		48	21	0.00010				
L-Leucyl-L-alaninamide				0.32	0.00029			6.0
L-Alanyl-L-leucine	0.0117	0.5	26	0.37	0.00058	0.5	21	6.0
		1	50	0.43		1	39	6.2
		1.5	65	0.43		2	64	6.4
		2	77	0.45				
L-Alanylglycine					0.00144			0.6
Glycyl-L-leucine	0.117			0.019	0.0029			0.35

two sensitive linkages are calculated as though the initial action is entirely on one bond.

D-Alanyl-L-leucinamide is hydrolyzed quite slowly; for Preparation B the rate is 900 times greater for the diastereoisomer, while for Preparation C it is 820 times greater. It appears likely that the hydrolysis of this compound occurs at the terminal amide linkage, *i.e.* the linkage at the L-leucine carbonyl rather than at the carbonyl of the D-amino acid residue. Although only slight hydrolysis of β-alanyl-L-leucinamide could be detected in 48 hours, similar results were obtained repeatedly. Here also it is probable that the terminal amide linkage is being split. The results indicate an almost absolute requirement for the α position of the free amino group. It may be noted that, since the relative rates with both preparations for the hydrolysis of the three isomeric dipeptide amides are similar, we must infer that the same enzyme attacks all of these compounds.

It had previously been assumed that leucine aminopeptidase had no significant action on glycyl-L-leucine, since marked alteration in the relative activities occurred on purification of extracts of mucosa when the hydrolysis of this peptide was compared to the rate for L-leucylglycine or L-leucinamide (4). In fact, a dipeptidase was later identified which hydrolyzes glycyl-L-leucine (14). However, we must now conclude that the aminopeptidase does have a slow but significant action on glycyl-L-leucine and that L-alanyl-L-leucine is hydrolyzed about twenty times more rapidly (average of Preparations B and C). For the same preparations, the data in Table I indicate that L-alaninamide is split nineteen times more rapidly than glycinamide. It is clear that substitution of L-alanine for the glycine moiety has the same effect on the rate of hydrolysis of both the dipeptides and the amides. This, of course, is to be expected from the rules of homospecificity elaborated by Bergmann and Fruton (15).

It is important to emphasize that, with crude tissue extracts, glycyl-L-leucine is hydrolyzed by both the aminopeptidase and the dipeptidase. Likewise, we have found that L-alanylglycine is hydrolyzed by the aminopeptidase (Table V). Nevertheless, as first pointed out by Linderstrøm-Lang (3), the main action of the crude intestinal extracts on this peptide is due to a distinct and exceedingly labile enzyme. We have found that this latter enzyme is not activated by Mn^{++} or Mg^{++} as is the aminopeptidase. Preliminary experiments with the alanylglycine-splitting enzyme of Preparation A have shown that the activity is inhibited by metal poisons such as cyanide and sulfide, and that activation is produced by Fe^{++} salts. It was also observed that the action of the crude Preparation A on L-alaninamide is not activated by Fe^{++}; this hydrolysis must be ascribed to leucine aminopeptidase. Thus, it appears from our present information that the main alanylglycine-splitting enzyme of the crude extract is not an aminopeptidase. Further study must await methods of stabilizing and purifying this enzyme.

Additional evidence that leucine aminopeptidase hydrolyzes L-alanyl-L-leucine and glycyl-L-leucine has been obtained by means of competition experiments involving the presence of two substrates simultaneously employing a low enzyme concentration (Table VI). If the two substrates were hydrolyzed by different enzymes, the amounts of hydrolysis would be additive. It is apparent that the rate of hydrolysis is that of the more sensitive compound in the mixture. Actually, the presence of the less sensitive compound appears to produce a slight inhibition. These results are explicable in simple kinetic terms only if zero order or a mixed zero and

TABLE VI

Hydrolysis of Substrate Mixtures

Each substrate was present at 0.05 M. The enzyme was Preparation C at 0.00029 mg. of protein N per cc. of test solution. The data are expressed as 100 per cent for the complete splitting of one peptide bond.

Time	Hydrolysis				
	L-Alanyl-L-leucine	L-Alanyl-L-leucine and L-leucinamide	L-Leucinamide	L-Leucinamide and glycyl-L-leucine	Glycyl-L-leucine
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0.5	10	10			
1	18	18			
1.5	27	25	19	12	
2.5	44	38	27	21	
6	75	75	49	40	10
18	100	148	88	88	34

first order reaction is involved. It is probable that the latter case holds here, although most of the data have been treated as indicating a first order reaction for present purposes.

DISCUSSION

From the data reported above and the previously available information concerning leucine aminopeptidase, it is now possible to evaluate a number of factors concerned in the specificity of this enzyme. It has been proposed that the enzyme-substrate complex of the metal-peptidases involves the formation of a compound in which the polar groups of the substrate are bound by the metal (16). For the aliphatic amino acid amides, we may assume that a minimum of 4 bonds of the metal is involved; 2 of these are linked to the protein and 2 to the substrate, while the side chain of the amino acid moiety is directly held by unknown forces of the protein. In the structure, the vertical dotted line indicates the point of cleavage.

R'
|
H C — N-H₂
| ⟩ Mn⁺⁺
C — N-|
‖   H₂ |
O

P
R
O
T
E
I
N
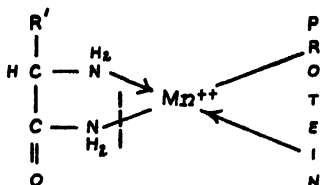
Evidence for complex formation between Mn^{++} and the substrate, L-leucylglycine, has already been detected in the marked change in absorption spectrum which occurs on mixing these two substances (17). Likewise, it has been found that the addition of Mn^{++} to highly purified preparations of leucine aminopeptidase produces a marked change in electrophoretic mobility of the proteins (13) and a striking change in the Mn^{++} spectrum (unpublished observations). Since such enzyme preparations are not homogeneous, such evidence is only suggestive that Mn^{++} is reacting with at least one component of the system to produce the change in color. Independent evidence of the reaction between Mn^{++} and the protein comes from the observation of a time reaction between metal and protein (4), and from the mass law character of the activation process (13).

It should be noted that we have assumed that the metal binds with the peptide nitrogen in this enzyme as already indicated for dipeptidases (16, 17). For carboxypeptidase, the evidence suggests that metal binding does not occur at the peptide nitrogen (16) since a peptide hydrogen is not essential (11); moreover, even the nitrogen is not essential, since carboxypeptidase possesses an exceedingly rapid esterase action (18). We have tested leucine aminopeptidase for esterase action by using L-leucine methyl ester as the substrate at pH 8.0. Only a slow saponification was found under the usual conditions of our enzyme experiments and the rate of this saponification was not influenced by the enzyme concentration. This indicates that for the action of this enzyme the peptide nitrogen is essential for the binding of the metal.

The structure of the enzyme-substrate complex shown for the amino acid amide indicates the formation of a 5-membered chelate ring involving the metal and the substrate. This is consistent with the usual strong complexes containing such a ring structure. The action of leucine aminopeptidase on glycyl-L-leucinamide (2), leading to a rapid liberation of ammonia followed by a slow hydrolysis of glycyl-L-leucine, poses a somewhat different problem. It is clear that the metal must bind with the free amino group which is essential (2), and with the amide nitrogen. If these were the only bindings, one would have to assume the formation of an exceedingly weak and improbable 8-membered ring. It is more likely that the metal forms an additional bond to the peptide nitrogen. This

would mean the presence of two 5-membered rings in which the weak bonds to the peptide and amide nitrogens are stabilized by the formation of ring
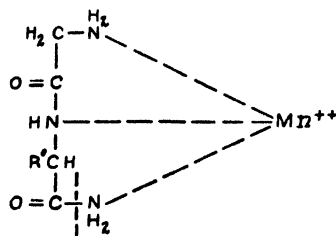
structures, as shown in the accompanying diagram. R' is the isobutyl side chain. This is analogous to the stabilization of ring structures in the complexes formed by aniline diacetic acid and ethylenediamine-tetraacetic acid with the alkaline earth metals which have been studied recently by Schwarzenbach and his collaborators (19).

It is clear that the aliphatic amides given in Table I differ only in the portion of the molecule (R') attached to the α-carbon.[2] In Fig. 1, there is plotted the relative value for the log C (taken from the averages of the data for the relative rates of hydrolysis of the aliphatic compounds in Table I for Preparations B and C) as a function of the size of the side chain as calculated from the molecular weight. Thus, for glycinamide, the molecular weight of the single hydrogen is one, while for alaninamide the CH_3 residue is 15, etc. The smooth curve shows a simple continuous hyperbolic function. The initial slope indicates that the rate increases about 10-fold for a 10-fold increase in mass. The curve also indicates that the maximal rate occurs with norleucinamide and leucinamide, which give identical values.

The changes in rates given for this series of compounds may be interpreted as due to changes in free energy of activation; these may be evaluated from the function $C \propto e^{-\Delta F/RT}$ or $\ln C \propto \Delta F/RT$, where C is the proteolytic coefficient, ΔF is the change in the free energy of activation, R is the gas constant, and T is the absolute temperature. (As a first approximation, we may assume that ΔS, the entropy of activation, is nearly the same for the various compounds.) For the increase in rate given by norleucinamide as compared with glycinamide, $\ln C_N - \ln C_G = -\Delta F/RT$,

[2] Errera and Greenstein (20) have found that crude aqueous extracts of normal and neoplastic tissues of rat and mouse hydrolyze the various aliphatic amino acid amides. Their results show considerable variability for the different compounds, depending on the tissue employed. Since it is likely that this is due to the presence of several different enzymes, we have not attempted any correlation of their data with those obtained for the purified aminopeptidase.

where ΔF is now the difference in free energy of activation for the hydrolysis of these two amides. The calculated value is about 3500 calories. The correlation of this value with the interaction between the protein and the hydrocarbon side chain of the amino acid amides will be considered in some detail elsewhere (21).

For certain peptidase actions, *e.g.* carboxypeptidase (16), it is apparent that the strength of the acid which is bound in the sensitive linkage has a
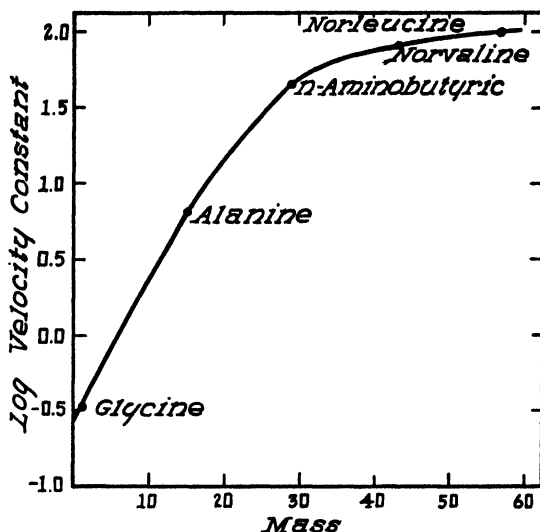
FIG. 1. Hydrolysis of the amides of aliphatic amino acids by leucine aminopeptidase. The logarithm of the relative first order velocity constant (average of the data for Preparations B and C given in Table I) is plotted as a function of the mass (molecular weight) of the side chain bound to the α-carbon. The value for L-leucinamide is identical with that plotted for norleucinamide.

great effect on the sensitivity of certain substrates towards the enzyme. For the series of aliphatic amino acid amides, it does not appear that this relationship has any significant influence. The various aliphatic amino acids all possess almost identical pK values (22); this suggests that the free energy of their amide bonds would also be identical.

The pronounced optical specificity exhibited by leucine aminopeptidase towards those compounds in which the configuration of the residue possessing a free amino group is altered indicates the applicability of the polyaffinity concepts described by Bergmann and his coworkers (8). This is apparent for an amino acid amide (D-leucinamide), for dipeptides (D-leucylglycine and D-leucyl-L-tyrosine), and for a dipeptide amide (D-alanyl-L-leucinamide). In each instance, the free amino group must be of the L

configuration for the simultaneous combination of the metal with the peptide nitrogen and the amino group, while the R group is bound directly to the protein. It appears, however, that the simple polyaffinity concept is not applicable in explaining the slower rate of hydrolysis of L-leucyl-D-alanine as compared to its diastereoisomer, L-leucyl-L-alanine, which is hydrolyzed at about the same rate as L-leucinamide and L-leucyl-β-alanine. This equivalence in rates suggests that the enzyme has little or no affinity for the residue bearing the peptide nitrogen in these compounds. The explanation given by Bergmann *et al.* (8) for the relatively rapid action on L-leucyl-D-alanine was predicated on the assumption that the responsible enzyme was a dipeptidase; this is not in keeping with the present information concerning the specificity of this enzyme. A possible explanation for the difference in rates for the diastereoisomeric peptides is that the β-methyl group of the D-alanine residue may interfere with the binding of the metal with the peptide nitrogen as suggested in the accompanying diagrams. Thus, this effect might be due to steric hindrance given by the relative positions of the methyl group and the carboxyl group in the D-alanine residue.

L-Leucyl-L-Alanine   L-Leucyl-D-Alanine
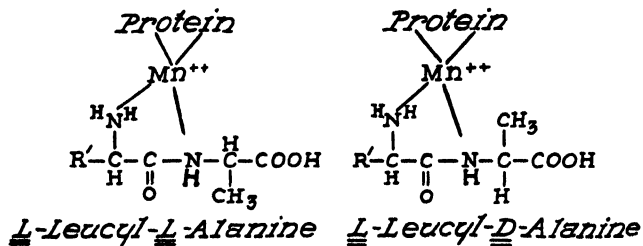
While the evidence discussed above indicates that the enzyme has little or no affinity for the residue possessing the peptide nitrogen, the high rate of hydrolysis of L-alanyl-L-leucine is in contradiction to this. Either another enzyme which possesses a rapid action on this peptide is present in these preparations, an assumption for which we have not been able to obtain any evidence, or the aminopeptidase possesses a great affinity for the leucine residue possessing the peptide nitrogen. If this affinity were similar to that exhibited towards the residue with the free amino group, the same relative rates would be expected for different terminal amino acids, as shown for the data in Fig. 1. This does not appear to be the case for the substrates tested up to the present.

SUMMARY

1. It has been found that, in addition to compounds like L-leucinamide, leucine aminopeptidase can also hydrolyze similar aliphatic L-amino acid amides. The rate of hydrolysis is markedly increased by an increase in the size of the aliphatic side chain attached to the α-carbon.

2. The enzyme possesses a marked specificity towards the residue possessing the free amino group. There is little or no hydrolysis of compounds like D-leucinamide, D-leucylglycine, D-alanyl-L-leucinamide, and β-alanyl-L-leucinamide. This is in accord with expectations from the polyaffinity theory.

3. It was observed that L-leucyl-L-alanine is split about twenty-three times faster than L-leucyl-D-alanine. This may be interpreted as due to an interference by the β-methyl group of D-alanine with the combination of the peptide nitrogen with the metal ion.

4. The action of this enzyme has been interpreted in terms of the formation of an intermediate enzyme-substrate complex in which the metal of the enzyme binds with the polar groups of the substrate, and the aliphatic side chains are held directly by the protein.

BIBLIOGRAPHY

1. Smith, E. L., and Polglase, W. J., *Federation Proc.*, **8**, 252 (1949).
2. Smith, E. L., and Slonim, N. B., *J. Biol. Chem.*, **176**, 835 (1948).
3. Linderstrøm-Lang, K., *Z. physiol. Chem.*, **182**, 151 (1929); **188**, 48 (1930).
4. Smith, E. L., and Bergmann, M., *J. Biol. Chem.*, **153**, 627 (1944).
5. Hanson, H. T., and Smith, E. L., *J. Biol. Chem.*, **175**, 833 (1948).
6. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).
7. Bergell, P., and von Wülfing, H., *Z. physiol. Chem.*, **64**, 348 (1910).
8. Bergmann, M., Zervas, L., Fruton, J. S., Schneider, F., and Schleich, H., *J. Biol. Chem.*, **109**, 325 (1935).
9. Fischer, E., and Steingroever, J., *Ann. Chem.*, **365**, 167 (1909).
10. Polglase, W. J., and Smith, E. L., *J. Am. Chem. Soc.*, in press.
11. Smith, E. L., *J. Biol. Chem.*, **175**, 39 (1948).
12. Behrens, O. K., and Bergmann, M., *J. Biol. Chem.*, **129**, 587 (1939).
13. Smith, E. L., *J. Biol. Chem.*, **163**, 15 (1946).
14. Smith, E. L., *J. Biol. Chem.*, **176**, 9 (1948).
15. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **145**, 247 (1942).
16. Smith, E. L., *Proc. Nat. Acad. Sc.*, **35**, 80 (1949).
17. Smith, E. L., *J. Biol. Chem.*, **176**, 21 (1948).
18. Snoke, J. E., Schwert, G. W., and Neurath, H., *J. Biol. Chem.*, **175**, 7 (1948).
19. Schwarzenbach, G., Willi, A., and Bach, R. O., *Helv. chim. acta*, **30**, 1303 (1947); Schwarzenbach, G., and Ackermann, H., *Helv. chim. acta*, **31**, 1029 (1948).
20. Errera, M., and Greenstein, J. P., *J. Nat. Cancer Inst.*, **8**, 71 (1947).
21. Smith, E. L., and Lumry, R., *Cold Spring Harbor Symposia Quant. Biol.*, **14**, in press.
22. Cohn, E. J., and Edsall, J. T., Proteins, amino acids and peptides, New York (1943).

THE FLUOROMETRIC DETERMINATION OF MALIC ACID*

By J. P. HUMMEL†

(From the Wenner-Gren Institute, University of Stockholm, Stockholm, Sweden, and the Department of Biochemistry, State University of Iowa, Iowa City)

A sensitive and specific chemical method for the estimation of malic acid in biological materials has long been desired. The condensation of malic acid with orcinol in the presence of concentrated sulfuric acid to form the highly fluorescent homoumbelliferone (7-hydroxy-5-methyl-coumarin) was reported years ago (1). Although this reaction is relatively specific for malic acid, the quantitative precipitation of calcium malate by alcohol as a means of fractionation from biological materials gives it additional specificity. If, in conjunction with this treatment, a small amount of 2,4-dinitrophenylhydrazine is added, most of the simple carbohydrate impurities which give an interfering amber color with orcinol become soluble in alcohol and thus may be removed.

Procedure

The solution to be analyzed is deproteinized with trichloroacetic acid of such a concentration that the final acidity is 1 N. Aliquots of the filtrate (containing 0.1 to 1.0 γ of malic acid) are transferred to 18 × 100 mm. test-tubes and are diluted to 1 ml. with 1 N HCl. To each tube are added with shaking 0.1 ml. of 0.1 per cent 2,4-dinitrophenylhydrazine in 2 N HCl and 0.5 ml. of 10 per cent $CaCl_2$ and, after 30 minutes, 0.3 ml. of 5 N NH_4OH and 6 ml. of absolute ethanol. After 12 hours at room temperature, the precipitation is complete. The tubes are strongly centrifuged and the supernatant is carefully poured off and discarded. To remove traces of moisture, the tubes are dried in the oven at 105° for 15 minutes.

A stock orcinol solution containing 80 mg. of orcinol (twice recrystallized from benzene) in 100 ml. of 12.5 per cent H_2SO_4 is prepared. This solution is stable for several months if stored in the cold in a brown bottle. To each tube, 3 ml. of orcinol-sulfuric acid mixture are added (8 ml. of stock orcinol solution diluted to 100 ml. with concentrated sulfuric acid of highest purity). The contents are mixed with bulb stirring rods (2).

The tubes are heated to 100° for 10 minutes, cooled under the tap, and diluted to 10 ml. with concentrated sulfuric acid. The blue fluorescence

is measured in a Coleman photofluorometer against blank and standard tubes treated in a similar manner. An almost linear fluorescence curve is obtained as shown in Fig. 1. The fluorometric attachment to the Beck-
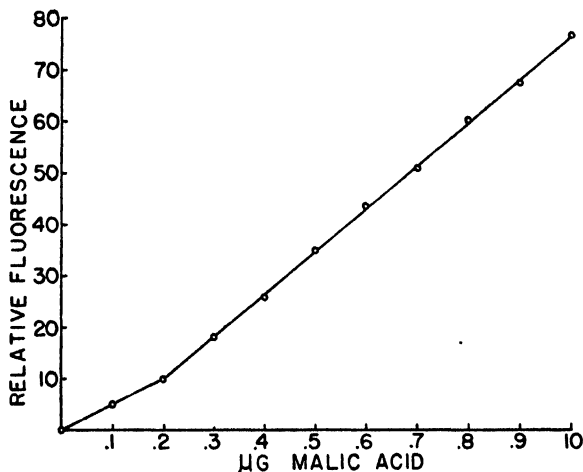
FIG. 1. The linear relation between the amount of malic acid assayed and the relative fluorescence produced.
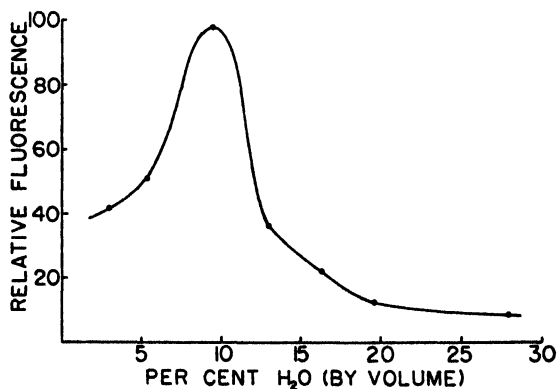
FIG. 2. The effect of different amounts of water in the orcinol-sulfuric acid mixture on the relative fluorescence produced by 1 γ of malic acid.

man spectrophotometer can also be used with somewhat greater concentrations of malic acid. The maximum fluorescence produced by 1 γ of malic acid is approximately equivalent to that given by 9 γ of quinine sulfate in 10 ml. of 0.1 N H_2SO_4. As shown in Fig. 2, the amount of water present during the condensation reaction is critical; less than 6 or more

than 12 per cent total water (by volume) in the sulfuric acid markedly reduces the fluorescence. It is therefore advisable to determine the water content of each batch of sulfuric acid.

TABLE I

Effect of Exercise on Content of Malic and Lactic Acids in Human Blood

Blood from finger-tips, 0.2 ml., was deproteinized with 6 ml. of 1 N trichloroacetic acid and centrifuged, and 1 ml. of the supernatant was treated as described in the text.

Subject No.	Malic acid	Lactic acid	Malic acid		Per cent recovery
			Added	Recovered	
At rest					
	mg. per cent	*mg. per cent*	γ	γ	
I	0.75	11			
II	0.75	15			
III	0.63	15			
IV	0.62	45			
V	0.62	35			
VI	0.36	17			
VII	0.42	18	0.50	0.50	100
VIII	0.28	11			
IX	0.39	5	0.81	0.81	100
X	0.24	15	0.81	0.83	102
XI	0.36	14	0.81	0.80	99
XII	0.24	20	0.85	0.86	101
XIII	0.33	31	0.85	0.81	95
Average.....	0.46	19			
After exercise					
IV	0.72	75			
V	0.59	85			
VI	0.51	53			
VII	0.37	86	0.50	0.50	100
XII	0.27	82	0.85	0.81	95
XIII	0.24	80	0.85	0.81	95
Average.....	0.45	77			

The following materials do not interfere at concentrations of 100 γ per tube: glucose, fructose, pyruvate, lactate, oxalacetate, isocitrate, aconitate, citrate, α-ketoglutarate, succinate, fumarate, β-hydroxybutyrate, acetoacetate, butyrate, tartrate, malonate, urate, glycerophosphate, aspartate, glutamate, alanine, and creatine. Samples of maleic acid tested gave

variable intensities of fluorescence, possibly due to contamination by malic acid. Fructose-1,6-diphosphate and glucose-6-phosphate do interfere since they are quantitatively precipitated by alcohol and produce both a yellow color and a blue fluorescence with the orcinol-sulfuric acid reagent. They may be easily removed from the sample by preliminary hydrolysis in 0.2 N NaOH for 10 minutes at 100°. Glycogen, which also interferes, may be removed by acid hydrolysis.

Malic Acid Content of Blood—As tested by this method, the levels of malic acid in finger-tip blood from normal male subjects were less than 1 mg. per cent, as shown in Table I, and did not change after exercise sufficiently vigorous to raise the blood lactic acid level to 3 or 4 times above the normal, as determined by the method of Barker and Summerson (3).

SUMMARY

A simple and sensitive fluorometric method for the determination of malic acid is described. The malic acid content of whole blood is low and does not change as a result of muscular activity.

BIBLIOGRAPHY

1. von Pechmann, H., and Welsh, W., *Ber. chem. Ges.*, **17**, 1646 (1884).
2. Vasseur, E., *Acta chem. Scand.*, **2**, 693 (1948).
3. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, **138**, 535 (1941).

A MICROMETHOD FOR ASSAY OF TOTAL TOCOPHEROLS IN BLOOD SERUM*

BY MARY L. QUAIFE, NEVIN S. SCRIMSHAW, AND OLIVER H. LOWRY

(From the Research Laboratory of Distillation Products, Inc., and the Department of Obstetrics and Gynecology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York, and the Department of Pharmacology, Washington University School of Medicine, St. Louis)

A simple, accurate micromethod for vitamin E in blood would be a desirable addition to assay procedures now available. There appears to be no vitamin E method which requires less than 2 to 5 ml. of serum or plasma, and this amount is frequently unobtainable from small children or experimental animals. Economy of space and time is another advantage of using a micromethod.

The micromethod for measuring vitamin E in blood to be described here appears to fit these requirements. A pair of analysts can do 80 assays in a working day (and carotene is also measured in the method). The volume of blood serum or oxalated plasma which is required (0.06 ml.) is easily obtained by finger-tip puncture. The method employs techniques previously described, whereby the Beckman spectrophotometer is adapted to measurement of optical density of very small volumes and micro pipettes of the Lang-Levy type are used (1, 2).

For determining vitamin E in blood the direct measurement of its absorption peak at about 295 mμ is attractive. However, owing to the very low extinction value of tocopherol and to the presence of many interfering substances which absorb in this region, this method is not practical. Of the oxidimetric color reactions for vitamin E, only two seem to be sensitive enough for use in a blood method. One is oxidation (e.g. with silver nitrate) to give tocopherylquinone with an absorption peak at about 265 mμ; it does not seem useful for microassay because of carotene interference and because of the rigid control required to obtain precise results. The other is the Emmerie and Engel (3) color reaction with ferric chloride and α,α'-dipyridyl to give a red color. Since the latter reaction is precise and easy to perform, it has been used.

The method is based on the macroprocedure of Quaife and Harris (4). However, the hydrogenation step, which obviates interference due to carotene, has been omitted since it was not found feasible to hydrogenate

---

* Communication No. 154 from the Research Laboratory of Distillation Products, Inc.

small samples without loss of tocopherol, presumably because the catalyst sensitizes it to oxidation. (In the hydrogenation apparatus used in the blood macromethod (5), this loss is prevented by use of a closed system, from which the air is rapidly swept out by a stream of hydrogen.) Instead, the light absorption due to the carotenoids is measured at 460 mμ and a correction made for the contribution of carotene to the Emmerie-Engel reaction color at 520 mμ.

Procedure

Reagents and Apparatus—

1. Absolute ethyl alcohol, purified as follows: Distil the alcohol from a flask containing pellets of potassium hydroxide and crystals of potassium permanganate. Discard the first portion.

2. Xylene, C.P.

3. α,α'-Dipyridyl in *n*-propyl alcohol, 0.120 gm. per 100 ml.

4. Ferric chloride hexahydrate in absolute ethyl alcohol, 0.120 gm. per 100 ml. Keep this solution in a dark brown or red glass bottle, fitted with a glass or cork stopper.

5. Pyrex test-tubes, 6 $\times$ 50 mm., fitted with cork (*not rubber*) stoppers.[1]

6. Lang-Levy constriction pipettes (2), 60, 40, and 13 c.mm. It is desirable to have the upper constriction quite small to facilitate pipetting organic solvents with low surface tension. (They can be purchased from the Microchemical Specialties Company, Berkeley 3, California.)

7. A Beckman spectrophotometer fitted with a micro attachment and 2 mm. quartz cuvettes (1). (The micro attachment and cells are obtainable from the Pyrocell Manufacturing Company, 207 East 84th Street, New York 28.)

8. Test-tube racks about 5 $\times$ 5 $\times$ 1½ inches to hold 100 tubes. These may be made from wire screen (two pieces of ½ inch mesh) or sheet metal.

9. A high speed hand drill, fitted as described by Bessey *et al.* (6).

Method

60 c.mm. of serum or plasma and 60 c.mm. of absolute ethyl alcohol[2] are put into a small (50 $\times$ 6 mm.) test-tube. The contents of the tube are mixed, immediately after the alcohol is added, by touching the side of

---

[1] Rubber stoppers are avoided because they contain substances readily extracted by organic solvents, which give a reaction with the Emmerie-Engel reagents. Corks are preferable, but contact with the solution must still be avoided. If small pieces of cork fall in the extraction mixture, false high Emmerie-Engel values will result. Corks covered with aluminum foil are satisfactory.

[2] All quantities of solution referred to in the method should be accurately measured. The exact amounts taken will depend on the volumes of the micro pipettes, and will not usually correspond exactly to the amounts stated.

the tube near the bottom to the whirling nail in the motor drill which has been mounted in a clamp with the nail up. 60 c.mm. of xylene are next added to each tube, and the tubes are corked to prevent loss by evaporation. Each tube is uncorked and mixed by touching to the motor drill long enough to allow violent agitation of the contents for at least 30 seconds, and then recorked. The tubes are centrifuged 10 minutes at 3000 R.P.M. To 40 c.mm. of the xylene supernatant layer, which has been transferred to another 50 $\times$ 6 mm. test-tube, are added 40 c.mm. of the α,α'-dipyridyl reagent. Each tube is touched to the motor drill to mix the contents and is then recorked. 60 c.mm. of the mixture are pipetted into a Beckman cuvette and the absorption read at 460 mμ with the use of distilled water as a reference blank. To the cuvette are then added 13 c.mm. of the ferric chloride reagent. The cuvette is rapidly rocked sideways through a 90° arc for 30 seconds to insure thorough mixing of the contents, and the absorption is measured at 520 mμ at 1½ minutes after the addition of the ferric chloride. Similar readings are made on blank solutions which have been prepared by applying all of the procedure described above for serum or plasma to an equal volume of distilled water. At least two blanks should be included for each Beckman cuvette. As with other micromethods, three Beckman cuvettes, each containing an individual sample, are used along with the reference cuvette which has distilled water.

It is imperative to clean the Beckman cuvettes prior to each color reaction by filling with acetone and sucking dry twice. Use of ether is avoided, as it frequently contains peroxides, which destroy tocopherol, and also impurities, which give a color with the Emmerie-Engel reagents.[3]

Calculation

Calibration data are based on pure, natural, d,α-tocopherol and on crystalline (90 per cent β-, 10 per cent α-) carotene. The tocopherol calibration curve is made from results obtained on solutions in absolute ethyl alcohol, ranging in concentration from 0.500 to 2.000 mg. per 100 ml. 60 c.mm. aliquots of these are added to equal volumes of distilled water and treated as described for blood. The carotene is dissolved in xylene (in a concentration range of 50 to 500 γ per 100 ml.), diluted 1:1 with the dipyridyl in propyl alcohol, and readings made at 460 mμ and also at 520 mμ after ferric chloride addition.

Calibration data should, of course, be determined by each individual, since slight deviations in technique frequently cause considerable varia-

---

[3] Pipettes and other glassware are cleaned in conventional fashion, e.g. with chromic acid. Then they are repeatedly rinsed with distilled water to remove any trace of acid or base which might affect the color reaction. Then they are rinsed with acetone and dried.

tions in results in a given analytical method. This is especially true for the color reaction employed here, since it gives increasing color with time. Once established, the calibration factor has remained surprisingly constant over a long period.

Under the conditions described and with the given volumes of solution, 57.4 c.mm. of xylene-dipyridyl reagent and 12.2 c.mm. of ferric chloride reagent, it was found that carotene gives a reading at 520 mμ in the Emmerie-Engel reaction, which is 29 per cent of that of carotene itself at 460 mμ. Therefore, readings on blood extracts at 520 mμ are corrected to this extent.

If in the standardization the same pipettes are used for standard α-tocopherol solution as for serum or plasma, the pipette volumes cancel out. When all values of standard and unknown are corrected for reagent blanks, the calculation for vitamin E (total free tocopherols) in plasma or serum becomes

$$\text{Mg. \% vitamin E} = \frac{(D_{520}-0.29D_{460})}{D_{520}\text{ of standard}} \times \text{mg. \% vitamin E in standard}$$

With use of the volumes given above, a value of 6.10 was determined for the ratio of mg. per cent of vitamin E of standard to D_{520} of the standard.

Carotene itself can, of course, be determined from the 460 mμ readings. (The formula determined in this laboratory is micrograms per cent of carotene $= 856 \times D_{460}$.)

DISCUSSION

On a macro scale the Emmerie-Engel reaction is preferably carried out with ethyl alcohol as the solvent, since rapid and complete color development occurs; also, possible color repression, which sometimes occurs when there is excess fat in the reaction mixture (7), is minimized. But it is too volatile for use in the micromethod. Acetic acid-petroleum ether mixtures as solvents have been avoided because they have the disadvantages mentioned above.

After some preliminary trial with various combinations of polar and hydrocarbon solvents, a 1:1 mixture of n-propyl alcohol and xylene was selected. Xylene is a hydrocarbon solvent which is sufficiently non-volatile for use in extracting vitamin E from serum in the micromethod. It must be diluted with at least an equal volume of polar solvent in order to give rapid and complete color development of the Emmerie-Engel reaction for tocopherol in a blood extract. The polar solvent chosen, n-propyl alcohol, is a compromise between the greater polarity of ethyl alcohol and the lesser volatility of the higher alcohols. (It has a boiling point 16° higher than that of isopropyl alcohol.)

Aliquots of a solution of carotene in a 1:1 mixture of *n*-propyl alcohol and xylene, when contained in micro Beckman cuvettes in minimum cell volumes, gave stable readings at 460 mμ for at least 30 minutes. The time required to read them in the actual blood procedure should not exceed 5 minutes.

Although the fact that a timed reaction is used on the Beckman instrument might appear to be a handicap, in practice little difficulty is encountered in obtaining readings, both on pure tocopherol and carotene and on blood samples, which show good reproducibility. This applies to readings made on successive samples in the three micro cuvettes (plus · the reference solution, distilled water) which are contained in the Beckman cuvette holder.

A large number of assays (*e.g.* 50 to 100) can be run simultaneously. Blanks must be run with each batch, but once the method is well standardized, standard solutions of vitamin E need not be run. The samples can be allowed to stand overnight in the refrigerator, after extraction and centrifugation, or after dilution of the xylene supernatant with the dipyridyl solution.

Tocopherol which had been added to the original serum extraction mixture at a level of 0.995 mg. per cent was recovered quantitatively (107 per cent) in the micromethod. (An absolute ethyl alcohol solution of pure α-tocopherol replaced ethyl alcohol in the extraction step.) This would be expected, since there are no steps in the procedure which are conducive to tocopherol loss, *e.g.* saponification.

Precision of the method appears as good as that of other micromethods. A few coefficients of variation are listed in Table I; they range from 2.7 to 5.7 per cent.

Results of assay by the micromethod were compared with those by the macromethod for a series of blood samples from presumably normal subjects, which include men and pregnant and non-pregnant women. They are listed in Table I. Good agreement is shown between them. The mean value for eighteen subjects was 1.11 mg. per cent by macroassay, and 1.07 mg. per cent by microassay.

Since the micromethod for vitamin E gives values identical with those given by the macromethod, within experimental error, the range of "normal" values would be the same: 0.8 to 1.2 mg. per cent for venous blood plasma or serum.

The micromethod was used to assay blood sera of rats which had been kept on a vitamin E-deficient diet for a year. The samples were obtained by cutting off the tip of the tail. The zero tocopherol levels found in the rat blood provide further evidence that extraneous materials other than tocopherol (and carotene) are not measured by the Emmerie-Engel reaction in the micromethod.

TABLE I

*Comparison of Total Tocopherol Content of Human Blood Serum As Determined by
Macro- and Microassay Methods*

Sample No.	Macro values, assayed in duplicate	Micro values		Coefficient of variation
		Concentration	No. of determinations	
	mg. per cent	mg. per cent		per cent
1	1.75	1.75 ± 0.06*	9	3.6
2	1.05	1.045 ± 0.028*	6	2.7
3	0.56	0.54 ± 0.03*	6	5.7
4	1.95	1.90	3	
5	0.73	0.71	3	
6	0.92	0.75	3	
7	0.76	0.76	3	
8	0.83	0.74	3	
9	0.97	0.92	3	
10	0.78	0.71	3	
11	1.33	1.26	3	
12	1.15	1.09	3	
13	1.39	1.21	3	
14	1.57	1.44	3	
15	1.05	1.03	3	
16	1.13	1.24	3	
17	0.91	0.87	3	
18	1.15	1.30	3	
Mean...........	1.11	1.07		

' Standard deviation.

TABLE II

Stability of Vitamin E in Blood Sera Stored in Deep Freeze at −22°

Sample No.	Tocopherols*		Loss
	Original	After 8 wks.	
	mg. per cent	mg. per cent	
1	1.90	1.73	9
2	0.54	0.54	0
Mean..			4.5

* Mean value of triplicate assay.

A preliminary check of the stability of vitamin E in blood sera on storage in the deep freeze has been made. The results (Table II) suggest that they are stable, within experimental error of the microassay, for at least 8 weeks when stored at −22°.

SUMMARY

A micromethod is described for determining total tocopherols in 0.06 ml. of blood serum or plasma, which consists of (1) precipitation of protein with ethyl alcohol and extraction of tocopherols into xylene; (2) dilution of an aliquot of xylene extract with α,α'-dipyridyl in n-propyl alcohol; (3) measurement of light absorption due to carotenoids at 460 mμ; and (4) addition of ferric chloride to the Beckman cuvette and measurement of absorption at 520 mμ.

The requirements of an analytical method with regard to precision, accuracy, and quantitative recovery of added tocopherol are satisfactorily met. Results agree well with those of the Quaife-Harris macroprocedure.

Blood sera stored at $-22°$ appear to be stable for at least 8 weeks with respect to tocopherol content.

BIBLIOGRAPHY

1. Lowry, O. H., and Bessey, O. A., *J. Biol. Chem.*, **163**, 633 (1946).
2. Bessey, O. A., Lowry, O. H., and Brock, M. J., *J. Biol. Chem.*, **164**, 321 (1946).
3. Emmerie, A., and Engel, C., *Rec. trav. chim. Pays-Bas*, **58**, 283 (1939).
4. Quaife, M. L., and Harris, P. L., *J. Biol. Chem.*, **156**, 499 (1944).
5. Quaife, M. L., and Biehler, R., *J. Biol. Chem.*, **159**, 663 (1945).
6. Bessey, O. A., Lowry, O. H., Brock, M. J., and Lopez, J. A., *J. Biol. Chem.*, **166**, 177 (1946).
7. Kaunitz, H., and Beaver, J. J., *J. Biol. Chem.*, **156**, 653 (1944).

PREPARATION OF SUCROSE LABELED WITH C14 IN THE GLUCOSE OR FRUCTOSE COMPONENT*

By H. WOLOCHOW, E. W. PUTMAN, M. DOUDOROFF, W. Z. HASSID, AND H. A. BARKER

(From the Division of Plant Nutrition, College of Agriculture, and the Department of Bacteriology, University of California, Berkeley)

The problem of sucrose utilization has not been sufficiently studied to ascertain whether the disaccharide is used directly, inverted by hydrolysis, or broken down through some other mechanism prior to further metabolism.

It is well known that fructose in its free form occurs as a relatively stable six-membered ring compound (pyranose configuration), whereas in the sucrose molecule it is found in the unstable five-membered ring form (furanose configuration), which cannot be isolated as a free substance. There is reason to suspect that this form, when liberated from sucrose by enzymatic action, might be metabolized in living cells in a different manner from the pyranose form or normal free fructose. The possibility also exists that the glucose half of the sucrose molecule, when first liberated, is phosphorylated and also reacts differently from free glucose in living organisms. However, it is difficult to distinguish the breakdown products of sucrose from one another in their final utilization by ordinary methods.

Labeling the components of sucrose with C14 affords a method for observing their metabolism in plants and animals. Since sucrose can be synthesized enzymatically from its constituent monosaccharides (1, 2), it is possible to label each half of the molecule by starting with monosaccharide units labeled with C14.

On the basis of earlier work the following reactions were postulated to account for the reversible phosphorolysis of sucrose with the enzyme from *Pseudomonas saccharophila* (2).

$$\text{Glucose-1-phosphate} + \text{enzyme} \underset{(a)}{\overset{\pm \text{ Phosphate}}{\rightleftarrows}} \text{Glucose-enzyme}$$

$$(b) \,\Big\updownarrow\, \pm \text{ Fructose}$$

$$\text{Glucose-1-fructoside} + \text{enzyme}$$
$$\text{(Sucrose)}$$
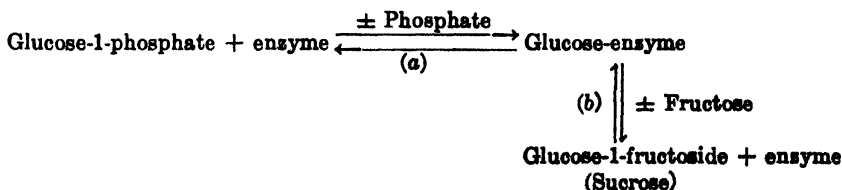
In reaction (a) phosphate is split off from the glucose-1-phosphate and the glucose residue combines with the enzyme forming a temporary bond, the glucose-enzyme combination existing in equilibrium with the glucose-1-phosphate. In the equilibrium reaction (b) the glucose from the glucose-enzyme complex is transferred to the fructose, which acts as an acceptor, forming sucrose.

These reactions were utilized to introduce labeled glucose into the sucrose molecule in one preparation and labeled fructose into this disaccharide in another.

Radioactive sucrose with "tagged" fructose was prepared by allowing radioactive fructose to exchange for fructose in the inactive sucrose molecule in the presence of an enzymatic extract from *P. saccharophila* according to the reaction

$$\text{Glucose-1-fructoside}^* + \text{enzyme} \rightleftarrows \text{glucose-enzyme} + \text{fructose}^*$$
$$\text{(Sucrose)}$$
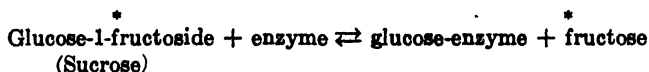
Sucrose labeled with C^{14} in the glucose moiety was obtained by allowing unlabeled fructose and sucrose to react with C^{14}-labeled glucose-1-phosphate by the aid of the same enzyme through the reversal of the phosphorolytic process

$$\text{Glucose-1-phosphate}^* + \text{fructose} \rightarrow \text{glucose-1-fructoside}^*$$
$$\text{(Sucrose)}$$

By hydrolyzing the synthetic radioactive sucrose preparations into their monosaccharide constituents, it was shown that in one sample 96 ± 3 per cent of the disaccharide activity resided in the glucose residue and less than 1 per cent in the fructose residue. In another sample, the fructose half of the molecule contained 90 ± 3 per cent of the activity, while the glucose contained less than 1 per cent. The probable error is only that due to variability in counting.

The incorporation of either radioactive glucose or fructose into the sucrose molecule illustrates one of the uses to which the enzyme, sucrose phosphorylase, may be put, and also confirms previous conclusions (2) concerning the mechanism of action of this enzyme.

<center>EXPERIMENTAL</center>

Reagents—The enzyme solution was prepared from 4 gm. of dried cells of *P. saccharophila* by fractional precipitation with ammonium sulfate from phosphate buffer at pH 6.64 and then from citrate buffer at pH 6.64 (2, 3).

C^{14}-labeled glucose-1-phosphate was prepared by McCready and Hassid's (4) method from a sample of radioactive starch, with use of potato phosphorylase. The radioactive starch was obtained from tobacco leaves that were exposed to an atmosphere of radioactive carbon dioxide and allowed to photosynthesize for about 24 hours (5).

The C^{14}-labeled fructose was obtained from similarly treated *Canna indica* leaves (5).

Sucrose with C^{14}-Labeled Fructose—The enzymatic digest consisted of 2.5 gm. of sucrose, 0.2 gm. of C^{14}-labeled fructose, and an extract from 2 gm. of dried cells from *P. saccharophila* in a total volume of 30 ml. of citrate buffer at pH 6.64. The digest was incubated for 6 hours at 30°, then pasteurized, and the remaining fructose fermented with a washed cell suspension of the yeast, *Torula monosa*. The yeast was removed by centrifugation and the electrolytes by the use of ion exchange columns, Duolite C-3 and Duolite A-3. The neutral solution was evaporated to 10 ml., 2.5 volumes of ethanol were added, and the alcoholic solution filtered. After evaporation of the solution, and treatment of the sirup with absolute alcohol, the sucrose was obtained in crystalline form. When the crude product was recrystallized twice from ethyl alcohol, its specific rotation in water ($c = 2$) was $[\alpha]_D = +66.5°$.

On the basis of the activity of the C^{14}-labeled fructose originally added, the radioactivity of the sucrose was found to be 76 per cent of the possible theoretical activity at equilibrium.

Resolution of Sucrose through Hypoiodite Oxidation and Determination of Activities of Monosaccharide Constituents—A 0.45 gm. sample of radioactive sucrose was hydrolyzed with 30 ml. of 1 N sulfuric acid for 10 minutes at 80°. The solution containing the invert sugar was neutralized with barium hydroxide, with use of phenolphthalein as an indicator, the barium sulfate was centrifuged off, and the supernatant liquid concentrated *in vacuo* to 10 ml. The glucose in this sample was oxidized to gluconic acid with BaI_2-I_2 solution according to a modified procedure of Goebel (6). After removing the barium and iodide impurities as described by this author, the solution was further purified by passing it through cation and anion exchange columns. The gluconic acid was adsorbed on the anion column, while the fructose passed through. The neutral solution containing the fructose was set aside. The gluconic acid was eluted from the column with 2 N ammonium hydroxide and the solution concentrated *in vacuo* to get rid of the excess ammonia. It was then passed again through the cation exchange column and the solution containing the gluconic acid was boiled with an excess of calcium carbonate, filtered while hot, and concentrated to a sirup. After the addition of a small amount of ethanol, the calcium gluconate crystallized out.

The product was recrystallized from water, washed with alcohol and ether, and dried at 50° *in vacuo*. A yield of 0.26 gm. was obtained. Its specific rotation in water (c = 2) was $[\alpha]_D$ = +9.0°. The specific rotation for calcium gluconate, $(C_6H_{11}O_7)_2Ca$, given by May, Weisberg, and Herrick (7) is 9.8°.

The neutral solution was concentrated to a sirup and taken up in a small amount of warm absolute alcohol. A few ml. of dry benzene were added, and the mixture was concentrated to dryness under reduced pressure, while a stream of dry air blew over the surface of the liquid. After repeating this procedure several times, the sirup was crystallized from absolute methanol by dropwise addition of anhydrous ether (8). The yield of fructose was 0.16 gm. The recovered fructose contained 95 per cent of the radioactivity of the sucrose, while that of the calcium gluconate contained 5 per cent.

Sucrose with C^{14}-Labeled Glucose—The reaction mixture consisted of 0.5 gm. of glucose-1-phosphate containing C^{14}; 0.2 gm. of inactive fructose and 2.5 gm. of inactive sucrose (carrier) were mixed with an extract from 2 gm. of dry cells from *P. saccharophila*. The reaction mixture was made up in a total volume of 30 ml. of citrate buffer at pH 6.64 and allowed to react for 6 hours at 30°. The radioactivity of the sucrose was found to be 72 per cent of the possible theoretical activity at equilibrium.

The isolated radioactive sucrose was resolved into its components by the method previously described. The recovered glucose, as calcium gluconate, contained 90 per cent of the activity of the sucrose, while the fructose contained 6 per cent.

The glucose component in the first sucrose preparation and the fructose component in the second sucrose preparation were expected to be inactive. The appreciable activity of 5 and 6 per cent found in the glucose and the fructose, respectively, was probably due to contamination. When glucose is oxidized in the presence of fructose, a small amount of the latter sugar is also oxidized. On the other hand, some of the glucose may remain unoxidized. In the process of separation, the non-radioactive gluconic acid is probably contaminated with the oxidized radioactive fructose, and the inactive fructose is contaminated with the unoxidized radioactive glucose.

A direct method for resolution of sucrose into its components, which does not require oxidation of the glucose with hypoiodite, was therefore tried.

Direct Resolution of Sucrose to Its Monosaccharide Constituents—1 gm. of finely powdered sucrose containing C^{14}-labeled glucose was treated in a centrifuge tube with 3 ml. of 95 per cent ethanol and 0.12 ml. of concentrated hydrochloric acid. The tube with the contents was kept at 50°

with frequent shaking until the sugar dissolved. The mixture was cooled to room temperature, seeded with 1 mg. of powdered anhydrous inactive glucose, and the tube stoppered and placed on a shaking machine. After several days the glucose crystallized out. The crystals were centrifuged, washed with absolute alcohol, and the crude product twice recrystallized from water by the addition of absolute alcohol. The yield of glucose was 0.270 gm. Its specific rotation in water ($c = 3$) was $[\alpha]_D = +52.5°$.

The acid-alcoholic mother liquor from the glucose crystallization was neutralized with silver carbonate, treated with charcoal, filtered, and concentrated to a sirup under a vacuum. The sirupy fructose was treated with hypoiodite to eliminate the remaining glucose, and was then further purified through calcium precipitation and decomposition of the complex with oxalic acid (5). By this procedure the fructose was obtained in crystalline form. The yield of fructose was 0.275 gm. Its specific rotation in water ($c = 3$) was $[\alpha]_D = -88°$.

Determination of Activity—The activity of the glucose and fructose was determined as follows: Approximately 10 mg. samples were burned in a semimicro combustion tube, and the carbon dioxide produced was trapped in 0.20 N carbon dioxide-free sodium hydroxide and precipitated as barium carbonate by the addition of 0.1 N barium chloride solution. The excess alkali was immediately titrated with 0.05 N hydrochloric acid. The barium carbonate obtained was washed in a centrifuge tube, suspended in ethanol, mounted on an aluminum disk, and assayed for radioactivity in a thin window Geiger tube.

The activity, in terms of the parent sucrose, of the glucose and fructose obtained from resolution of the two sucrose samples as outlined by the latter method was as follows: glucose, 0.7 ± 0.3 per cent; fructose, 0.6 ± 0.3 per cent.

SUMMARY

With use of C^{14}-radioactive fructose, inactive sucrose, and an enzymatic extract from *Pseudomonas saccharophila*, sucrose with a C^{14}-labeled fructose component was synthesized.

Sucrose with C^{14}-labeled glucose was prepared by allowing labeled glucose-1-phosphate to react with inactive fructose in the presence of the same enzyme.

Analysis of the first synthetic sucrose preparation showed that practically all of the disaccharide activity could be accounted for by the fructose part of the molecule, while the glucose contained only 0.7 per cent of the activity. Resolution of the second sucrose preparation and analysis of its components showed essentially all of the activity in the glucose and 0.6 per cent in the fructose.

BIBLIOGRAPHY

1. Hassid, W. Z., Doudoroff, M., and Barker, H. A., *J. Am. Chem. Soc.*, **66**, 1416 (1944).
2. Doudoroff, M., Barker, H. A., and Hassid, W. Z., *J. Biol. Chem.*, **168**, 725 (1947).
3. Doudoroff, M., *J. Biol. Chem.*, **151**, 351 (1943).
4. McCready, R. M., and Hassid, W. Z., *J. Am. Chem. Soc.*, **66**, 560 (1944).
5. Putman, E. W., Hassid, W. Z., Krotkov, G., and Barker, H. A., *J. Biol. Chem.*, **173**, 785 (1948).
6. Goebel, W. F., *J. Biol. Chem.*, **72**, 809 (1927).
7. May, O. E., Weisberg, S. W., and Herrick, H. T., *J. Washington Acad. Sc.*, **19**, 443 (1929).
8. Fischer, H. O. L., and Baer, E., *Helv. chim. acta*, **19**, 519 (1936).

THE EFFECT OF HEAT ON THE AVAILABILITY OF PORK PROTEIN IN VIVO AND IN VITRO

By J. F. BEUK, F. W. CHORNOCK, AND E. E. RICE

(From the Research Laboratories, Swift and Company, Chicago)

In earlier work it has been shown that prolonged autoclaving of pork muscle does not destroy significant quantities of essential amino acids, although it does reduce the amount of cystine in the meat (1). This observation was based upon amino acid analyses of acid hydrolysates of raw and autoclaved meat. However, it was further shown that the severe heat treatments used greatly decreased the extent to which trypsin and erepsin could release the amino acids from pork protein. Thus, decrease in availability rather than amino acid destruction seems a more probable cause of the lowering of biological values which various investigators have found.

Since these previous experiments as well as others in the literature demonstrated that prolonged heat treatment reduced the enzyme digestibility of meat *in vitro* as well as its biological value, it became of interest to know the effects of less severe treatments. It was hoped that these studies might reveal some correlation between enzyme digestibility and biological value and also indicate the readiness with which heat affects the digestibility of meat. Clandinin (2) has already demonstrated some similarity between enzyme digestibility of fish meals and their biological value for chicks.

EXPERIMENTAL

Preparation of Samples

A 20 pound quantity of fresh pork shoulder was trimmed of fat, comminuted in a high speed chopper, and mixed several times to assure uniformity. The meat was packed into thermal death time cans[1] and sealed under vacuum in a commercial type of closing machine. Several cans were autoclaved at 113° for each of the following time intervals: 5, 15, and 30 minutes and 1, 2, 4, 8, and 24 hours. Upon the completion of each autoclaving period, the cans were cooled immediately and stored, along with the raw controls, at −20° until needed. This procedure was repeated when larger quantities of sample were needed for the biological experiments.

[1] These cans measure 2½ × 5/16 inches. They were kindly furnished by J. F. Feaster of the American Can Company, Maywood, Illinois.

Assay Procedure

Enzyme digests and acid hydrolysates of the various samples were prepared as previously described (1). In brief, this amounted to treatment of the samples with trypsin and erepsin for periods of 48 hours at pH 7.8. In each case, the final dilutions corresponded to 5 gm. of sample per 100 ml.

Aliquots of each suspension were treated with 0.1 N sulfuric acid and later analyzed for total nitrogen by the micro-Kjeldahl procedure. Other aliquots were treated with sodium tungstate and sulfuric acid, filtered, and analyzed to obtain values for non-protein nitrogen and amino acid

TABLE I

Composition of Typical Rat Diet

Ingredients	Quantities added
	gm.
Sucrose	15.00
Starch	51.01
Meat*	12.49
Fat†	14.70
Salts‡	4.00
Vitamin A and D oil (3000 A, 500 D)	1.00
Wheat germ oil (1 mg. tocopherol per gm.)	1.00
Choline	0.10
Vitamin mixture§	0.71

* The meat, either raw or cooked, supplied 9 per cent of protein and 3.3 per cent of the fat.

† The total fat, including meat fat, was 18.00 per cent.

‡ Jones-Foster salt mixture (5).

§ Thiamine hydrochloride 0.8 mg., riboflavin 1.6 mg., niacin 4.0 mg., pyridoxine hydrochloride 0.8 mg., calcium pantothenate 4.4 mg., liver concentrate, Wilson's (1:20) 700 mg., and 2-methyl-4-naphthoquinone, 1.0 mg.

nitrogen. The remainder of the sample solutions was filtered and determinations of free amino groups and of free amino acids were made upon each of these filtrates. The Koch modification (3) of the nitrous acid volumetric method of Van Slyke and the ninhydrin manometric method of Van Slyke, Dillon, MacFadyen, and Hamilton (4) were used in the respective analyses.

Microbiological assays of each filtered sample were made for the amino acids listed in Table III by the microbiological technique previously described (1).

Bioassay with Rats

In order to test the biological availability of the proteins given different heat treatments, four diets at a 9 per cent level were prepared: one

each of the raw meat, and meat autoclaved 5 minutes, 2 hours, and 24 hours. Cystine in an amount equal to that found in the raw meat was added to part of the diet containing the meat autoclaved 24 hours. Table I shows the composition of a typical diet used in the feeding experiment, listing quantities of each ingredient for 100 gm. of diet (dry weight).

Male albino rats weighing approximately 50 gm. were obtained from the Carworth Farms and placed on stock ration for several days. The gain in weight of each was recorded and animals showing unusually low or high gain in growth were discarded. Those remaining were placed in individual cages and divided into four groups of eight each and one group of four.

TABLE II

Effect of Autoclaving Pork at 115° upon Liberation of Various Nitrogen Fractions by Trypsin and Erepsin

Methods of analysis	N liberated per gm. raw sample	N expressed as per cent of N in raw sample							
		Autoclaving time							
		5 min.	15 min.	30 min.	1 hr.	2 hrs.	4 hrs.	8 hrs.	24 hrs.
	mg.								
Total N of tungstic acid filtrates	19.7	93.0	91.0	89.9	88.7	85.5	81.7	74.7	76.7
Free amino acid* N of tungstic acid filtrates	8.1	60.2	59.6	56.2	56.7	55.5	55.8	54.7	50.5
Free amino acid* N of untreated filtrates	11.9	47.9	50.4	47.5	43.8			37.8	
Free amino N† of untreated filtrates	17.5	68.5	68.2	69.9	65.2	63.2	59.1	59.4	46.7

* Determined by the Van Slyke-Dillon-MacFadyen-Hamilton procedure (4).
† Determined by the Van Slyke volumetric procedure, Koch modification (3).

The average weights for the groups and their responses to the various diets are shown in Table V.

DISCUSSION

The rate of liberation of nitrogen by enzymatic digestion of the raw and autoclaved pork is shown in Table II. The non-protein nitrogen values show a relatively slow decline over the entire period of autoclaving. The amino nitrogen values, determined by the nitrous acid method which includes the free ε-amino nitrogen in addition to the free α-amino nitrogen, declined rapidly during the first 5 minute interval and more slowly thereafter during the 24 hour period. These results were confirmed by the values obtained for free amino acid nitrogen by use of ninhydrin. Though the initial nitrogen values obtained by the two methods show wide differences, the same relative rates of decline in the liberation of nitrogen were

maintained during the additional periods of autoclaving. This suggests that the major change in the protein occurs very rapidly, and that subsequent changes are slow and small.

Although the extent of the release of each of the various amino acids by enzymes (Table III) decreases with increasing duration of the heat treatment of the protein, the rate of release of the amino acids varied widely. Thus, based on the amount released from the raw pork, the amounts of the individual amino acids liberated from pork autoclaved for 5 minutes ranged from 96.6 per cent for arginine to 41.7 per cent for cystine. For the 24 hour autoclaving period, the release varied from 66.8 per cent

TABLE III

of Autoclaving Pork at 113° upon Liberation of Amino Acids by Trypsin and Erepsin

Amino acid	Amino acids liberated per gm. raw sample	Amino acids expressed as per cent in raw sample							
		Autoclaving time							
		5 min.	15 min.	30 min.	1 hr.	2 hrs.	4 hrs.	8 hrs.	24 hrs.
	mg.								
Arginine...................	8.4	96.6	96.5	92.8	88.0	87.0	83.2	74.0	66.8
Leucine.........	14.2	96.5	94.5	83.5	74.5	67.5	67.0	64.5	64.0
Valine........	7.8	89.9	85.5	78.3	70.5	70.5	65.5	60.5	56.5
Isoleucine..	11.0	91.0	83.6	83.6	77.2	71.7	65.4	59.0	55.5
Lysine...	16.2	89.5	81.5	74.7	72.2	69.0	64.8	59.6	53.6
Phenylalanine.	5.9	83.0	79.6	78.0	74.6	71.3	64.5	62.8	52.5
Threonine......	8.7	81.6	78.3	73.5	67.9	63.3	57.5	56.5	51.7
Tryptophan	2.1	71.5	62.0	57.2	57.2	52.4	52.4	52.4	47.5
Methionine.....	4.2	64.3	59.5	54.8	50.0	50.0	47.6	45.3	42.8
Histidine......	6.5	62.8	59.0		53.3	54.3	46.5	40.4	38.7
Cystine.........	1.6	41.7	35.0	25.0	18.7	14.4	8.2	5.0	1.2

for arginine to 1.2 per cent for cystine. The same relative extent of release was maintained for most of the amino acids regardless of the length of the autoclaving period. From these data it may be surmised that the utilization of the autoclaved protein may be less than that of the fresh because of the low availability of certain essential amino acids and because of their unequal rates of liberation. For maximum utilization of the protein, all of the essential amino acids should be available at the same time (Melnick and Oser (6)).

The occurrence of much larger amounts of amino nitrogen than of free amino acid nitrogen (nitrous acid *versus* ninhydrin values) suggests the presence of soluble peptides in the filtrates of the autoclaved samples. These are not readily diminished in quantity by prolonged enzyme treat-

ment; hence they may be assumed to be difficultly digestible by those enzymes which are capable of more completely converting the raw pork protein into free amino acids. The significance of such soluble but unavailable peptides may be observed by studying the distribution of amino acids and amino nitrogen in various fractions of an enzyme-digested, cured, autoclaved pork sample (Table IV). The total amino acid content of the enzyme-digested sample is shown in Row 1, as the acid-treated meat digest. By filtration the suspension can be divided into the soluble and insoluble fractions, for which the amino acid contents are given in Rows 4 and 6, respectively.

The filtrate of the enzyme-treated pork·gives much lower amino acid values (Row 4) than the same filtrates after acid treatment (Row 3), which is more evidence for a soluble but unavailable form of amino acid-containing compound. Row 5 lists the difference between the untreated filtrate (Row 4) and the acid-treated filtrate (Row 3), and, theoretically, represents the amount of amino acid in the soluble but unavailable compound. Although values obtained by difference are less satisfactory than those obtained by direct analysis, they are the only ones available until a separation of the free and bound amino acids can be effected. The magnitude of the values in Row 5, even if allowance is made for considerable experimental error, is sufficient to indicate that the composition of the soluble but unavailable fraction differs markedly from either that of the original protein or that of the available amino acids. This form represents more than 50 per cent of the methionine and 40 per cent of the cystine and threonine, but less than 10 per cent of the leucine and less than 20 per cent of the arginine, isoleucine, and valine. It is thus rich in methionine and cystine, two of the nutritionally important amino acids. It is possible that peptides not available to the microorganisms may not be accessible also to the higher forms of life; hence efforts were made to correlate values of digestibility *in vitro* with those *in vivo*.

The effect of heat treatment of pork on the weight of the rats fed diets containing either the raw or one of the autoclaved meat samples is presented in Fig. 1. Rats receiving the raw meat diet, Group I, showed the greatest gain in weight and the highest protein efficiency. This was to be expected since studies *in vitro* show the raw meat to be the most highly digestible. The rats fed the diet containing meat autoclaved 5 minutes, Group II, showed lower weight gains than did Group I, but the protein efficiencies were similar, being 3.2 and 3.0 for Groups I and II, respectively. Statistical analyses of the data indicated the differences in growth to be significant at the level of $P = 5$ per cent but not at $P = 1$ per cent. There were no significant differences between the protein efficiencies of these two groups (Table V).

TABLE IV

Distribution of Free Amino Nitrogen and Amino Acids in Meat Digests and Filtrates, before and after Acid Hydrolysis and in Acid-Treated Precipitate

The results are expressed in mg. per gm. of sample. The values in parentheses represent the per cent amino nitrogen and amino acids liberated, based on the values obtained for the acid-treated meat digest.

Row No.	Amino N	Arginine	Cystine	Histidine	Isoleucine	Leucine	Lysine	Methionine	Phenyl-alanine	Threonine	Valine
1	21.2 (100)	6.8 (100)	2.5 (100)	5.7 (100)	8.7 (100)	11.9 (100)	12.1 (100)	3.8 (100)	6.8 (100)	7.8 (100)	7.7 (100)
2	13.2 (62.2)	4.9 (72.0)	1.4 (56.0)	4.3 (75.5)	6.0 (69.0)	9.0 (75.6)	8.6 (71.0)	2.1 (55.3)	4.5 (66.2)	3.4 (43.5)	5.9 (76.6)
3	21.0 (99.0)	5.3 (78.0)	2.2 (88.1)	4.7 (82.5)	6.9 (79.4)	9.0 (75.6)	11.1 (91.8)	3.6 (94.8)	6.2 (91.2)	6.4 (82.0)	5.9 (76.6)
4	8.7 (41.0)	4.3 (63.3)	1.3 (52.0)	3.4 (59.6)	5.7 (65.5)	8.3 (69.7)	7.7 (63.7)	1.7 (44.6)	4.4 (64.8)	3.4 (43.5)	5.3 (68.8)
5	12.3 (58.0)	1.0 (14.7)	0.9 (36.0)	1.3 (22.8)	1.2 (13.8)	0.7 (5.9)	3.4 (28.1)	1.9 (50.0)	1.8 (26.5)	3.0 (38.5)	0.6 (7.8)
6	0.9 (4.3)	1.1 (16.2)	0.2 (8.0)	0.7 (12.3)	1.6 (18.4)	2.6 (21.8)	1.1 (9.2)	0.4 (10.5)	0.7 (10.3)	1.5 (19.2)	1.2 (15.6)

Row 1, enzymatic digest; Row 2, enzymatic digest, acid-treated; Row 3, filtrate of enzymatic digest; Row 4, "unavailable" amino acids (obtained by subtracting Row 4 from Row 3); Row 5, filtrate of enzymatic digest, acid-treated; Row 6, precipitate from enzymatic digest, acid-treated.

Group III and Group IV, fed diets containing meat which had been autoclaved for 2 and 24 hours, respectively, showed even smaller gains and protein efficiencies. When compared to Group I, significant differences were obtained for these two groups. The addition of cystine to one of the diets containing meat which had been autoclaved for 24 hours, Group V, improved the diet slightly but did not restore it to normal.
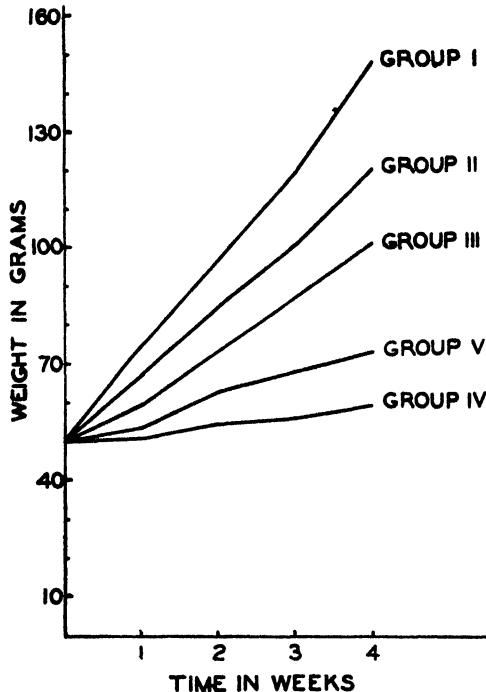
FIG. 1. Growth rates of rats

Clandinin (2) and Seegars and Mattill (8), using herring meal and beef proteins, respectively, showed by digestibility studies, both *in vitro* and *in vivo*, that the severely heated products were inferior to the corresponding raw samples. Their data are in agreement with those presented in this paper.

No exact correlation can be drawn between the studies *in vitro* and *in vivo*, inasmuch as the former method has a tendency to exaggerate the heat effect during the short autoclaving intervals. In general, however, those samples showing the least digestibility *in vitro* are of the lowest biological value. While these data indicate general effects of heat upon digestibility *in vitro* and on the biological value of meat, it should be re-

membered that the treatment of most of the samples far exceeded the heating which is normally given meat. Since thin cans were used, essentially all of the meat was at the elevated temperatures for the full period of heating. Even under these conditions, autoclaving had to be continued for 2 hours before a clear cut decrease in protein efficiency was observed. Moreover, in the experiments *in vivo*, the rats fed the diets containing meat autoclaved for 2 or 24 hours consumed approximately half as much food as those fed the raw meat diet. For this reason it would

TABLE V

Evaluation of Biological Data

Group No.	No. of rats in group	Average initial weight*	Average gain	Standard deviation	t†	Significant difference between average gains	Average quantity of protein consumed	Protein efficiency	Standard deviation	t†	Significant difference between efficiencies
		gm.	*gm.*				*gm.*				
I	8	49.8	98.8	27.8			30.4	3.2	0.32		
II	8	49.6	70.8	21.8	2.24	Yes at 5% No " 1%	23.9	3.0	0.57	0.086	No at 5%
III	8	50.0	52.9	16.9	3.99	Yes " 1%	17.8	2.6	0.37	3.68	Yes " 1%
IV	8	49.5	9.6	7.2	8.75	" " 1%	14.3	0.7	0.38	14.40	" " 1%
V	4	49.8	23.0	2.7	7.40	" " 1%	15.9	1.4	0.21	9.90	" " 1%

Group I, raw meat; Group II, meat autoclaved 5 minutes; Group III, meat autoclaved 2 hours; Group IV, meat autoclaved 24 hours; Group V, meat autoclaved 24 hours plus cystine.

* The duration of the experiment was 4 weeks.

† Data from each group were compared to those of the raw meat and premix group to obtain "Student's" t (Snedecor (7), p. 77). When there was a significant difference between variance, modified t tests were employed (Snedecor (7), p. 83).

appear entirely feasible to conclude that decreased palatability of the product might be the reason for poor growth instead of or in addition to decreased digestibility.

SUMMARY

When non-protein nitrogen, the nitrogen of the free amino groups and of free amino acids, and the individual amino acid assays were taken as criteria of digestion, it was shown that increasing the periods of autoclaving from 5 minutes to 24 hours caused a progressive decrease in the digestibility of pork by trypsin and erepsin *in vitro*. The non-protein nitrogen showed a slow but steady decline throughout the entire series of tests. The free amino nitrogen and the free amino acid nitrogen showed a sudden drop in the digestibility of pork for the initial period, with a gradual decline thereafter.

There was much variation in the extent to which the amino acids could be liberated by enzymes from the autoclaved pork. The liberation ranged from 96.6 per cent for arginine and 41.7 per cent for cystine for the 5 minute period to 66.8 per cent for arginine and 1.2 per cent for cystine for the 24 hour period. Except for arginine and leucine, the largest percentage decrease of amino acids liberated occurred during the first 5 minutes of autoclaving, indicating that the effect of heat is rapid.

The existence of soluble but unavailable peptides is indicated, since acid hydrolysis of filtered enzyme digests of autoclaved meat increased the amounts of amino acids available to the test microorganisms. These peptides contain appreciable quantities of certain essential amino acids.

Rats fed a raw meat diet showed the largest average weight gain and protein efficiency, followed in order by rats fed diets containing meat which had been autoclaved for 5 minutes, 2 hours, and 24 hours. The addition of cystine to the diet containing meat which had been autoclaved for 24 hours resulted in only a slightly higher gain in weight and protein efficiency.

BIBLIOGRAPHY

1. Beuk, J. F., Chornock, F. W., and Rice, E. E., *J. Biol. Chem.*, **175**, 291 (1948).
2. Clandinin, D. R., *Poultry Sc.*, **28**, 128 (1949).
3. Koch, F. C., *J. Biol. Chem.*, **84**, 601 (1929).
4. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).
5. Jones, J. H., and Foster, C., *J. Nutr.*, **24**, 245 (1942).
6. Melnick, D., and Oser, B. L., *Food Tech.*, **3**, 57 (1949).
7. Snedecor, G. W., Statistical methods, Ames 4th edition (1946).
8. Seegars, W. H., and Mattill, H. A., *J. Nutr.*, **10**, 271 (1935).

USE OF COPPER (II) ION IN MASKING α-AMINO GROUPS OF AMINO ACIDS

BY ALTON C. KURTZ

(From the Department of Biochemistry, University of Oklahoma School of Medicine, Oklahoma City)

The use of ornithine copper in preparing DL-citrulline suggested (1) the wider application of chelation to synthetic work. Although ions other than copper which are capable of forming chelate rings with α-amino acids also mask the coordinated groups, the use of such ions ordinarily cannot be expected to be advantageous. The superiority of the cupric ion over others, such as cobalt (III) ion, lies in the ease with which stable complexes may be formed and in the facility with which the copper may be removed subsequently. The stable cupric complexes of amino acids may be formed simply by heating aqueous solutions of the latter with an excess of cupric carbonate, cupric oxide, or cupric hydroxide, but tests with cobalt demonstrated the troublesome necessity of oxidizing the cobalt to the trivalent state. The extremely low solubility of cupric sulfide, even in acidic solutions, permits the ready removal of copper from all of its complexes by treatment with hydrogen sulfide.

Three diamino acids have been studied. Ornithine and lysine were selected because of their availability and because the reacting, terminal, amino groups in these compounds are structurally identical with the groups it was desired to mask at the α position. This structural identity of the α- and ω-amino groups should make tests of the masking ability of the cupric ion the more severe. The amino groups of α,γ-diaminobutyric acid are sufficiently close together to permit, theoretically, the simultaneous coordination of both reactive groups with a single cupric ion. If a chelate ring containing the 2 nitrogen atoms were formed, masking of both might be expected. To determine whether such masking actually occurs, α,γ-diaminobutyric acid was also studied.

EXPERIMENTAL

Carbamyl Derivatives—The procedures were analogous to that previously described (1) for the preparation of DL-citrulline except that the amino acid solution, adjusted, when necessary, to pH 7 with sodium hydroxide, was boiled with basic cupric carbonate rather than with cupric oxide. Excess cupric carbonate is more readily removed by filtration than is the oxide.

L-*Citrulline* (*2*)—This was obtained in 78 per cent yield from the copper complex (57 per cent yield) prepared from L-ornithine dihydrochloride. The active citrulline, which has a melting point of 221–222° (all melting points corrected) with decomposition and rotation unchanged by five recrystallizations from water plus ethanol, gave $[\alpha]_{5461}^{23}$ = +4.3° (c = 0.20 M in water) and $[\alpha]_{5461}^{23}$ = +25.9° (c = 0.20 M in 2.0 M hydrochloric acid).

Analysis—$C_6H_{13}N_3O_3$.   Calculated, N 23.98; found, N 23.8

L-Citrulline copper prepared from the amino acid by precipitation with cupric acetate solution turned gray above 210° and melted at 249–250° (decomposition).

DL-*Homocitrulline*[1]—This was prepared from DL-lysine dihydrochloride in 57 to 67 per cent over-all yields.[2]   Yields were highest when urea and lysine copper chloride were heated in a sealed bomb at 103–105° for 5 hours; after 18 hours heating, the yield fell to 36 per cent.   DL-Homocitrulline is faintly sweet and very soluble in water and insoluble in alcohol and in ether.   Crystals were elongated leaflets and melted at 222–224° with gas evolution.

Analysis—$C_7H_{15}N_3O_3$
     Calculated.   C 44.43, H 7.98, N 22.20, amino N 7.40
     Found.   " 43.3,   " 7.88,   " 21.3,   "   " 7.5

Aqueous cupric acetate added to a solution of the homocitrulline gave microscopic pale blue birefringent needles of DL-homocitrulline copper, which, dried over sulfuric acid, turned gray above 225° and melted at 258° (decomposition).

Analysis—$(C_7H_{14}N_3O_3)_2Cu$.   Calculated, Cu 14.44; found, Cu 14.46

L-*Homocitrulline*—The copper complex was prepared from L-lysine dihydrochloride in 80 to 86 per cent yields.[3]   The filtrate from cupric

[1] This compound, ε-carbamyllysine, is mentioned in British patent 421,407 (1934), but is there described merely as having properties "similar to those of delta-carbamido-ornithine."

[2] This was for DL-lysine dihydrochloride prepared according to Eck and Marvel (3).   Several commercial samples of lysine salt gave slightly lower yields and a single light brown sample of unknown history gave only dark solutions with pyridine-like odors from which no homocitrulline could be isolated.   Thus it is evident that some commercial samples of lysine formerly supplied were of dubious purity.   One sample of active lysine dihydrochloride purchased in 1937 contained large amounts of histidine as shown by its strong reaction with p-diazobenzene-sulfonic acid.

[3] These yields were obtained on various domestic samples of active lysine dihydrochloride; an imported sample gave only about 30 per cent yields.

sulfide was evaporated to dryness *in vacuo*. The L-homocitrulline was taken up in a little warm water and precipitated by the gradual addition of several volumes of acetone. Gels resulted if too little acetone was present in the final solutions. Yields of active homocitrulline from the copper complex averaged 94 per cent. Recrystallization from aqueous acetone gave aggregates of platelets, m.p. 211–212° (decomposition). Rotations were $[\alpha]_{546}^{25} = +6.9°$ ($c = 0.20$ M in water) and $[\alpha]_{546}^{25} = +27.5°$ ($c = 0.10$ M in 1.0 M hydrochloric acid).

Analysis—$C_7H_{15}N_3O_3$. Calculated, N 22.20; found, N 21.3

The copper complex, from cupric acetate and a dilute solution of L-homocitrulline, turned gray above 220–225° and melted at 246–247° (decomposition).

Analysis—$(C_7H_{14}N_3O_3)_2Cu$. Calculated, Cu 14.44; found, Cu 14.49

L-α-*Amino-γ-carbamidobutyric Acid*—This compound, next lower homologue of citrulline, was isolated as the copper derivative in yields of 35 to 69 per cent in runs of 0.005 to 0.03 mole when L-α,γ-diaminobutyric acid dihydrochloride[4] was submitted to the sealed tube procedure. L-α-Amino-γ-carbamidobutyric acid (yield 87 per cent from the complex) gave slightly sweet, colorless, elongated platelets from aqueous ethanol and melted at 225° with gas evolution. The compound was very soluble in water and insoluble in ethanol, ether, and acetone. A 0.20 M aqueous solution gave a rotation too small to read accurately, but, for a 0.20 M solution in 2.0 M hydrochloric acid, $[\alpha]_D^{30} = +27.9°$ and $[\alpha]_{546}^{30} = +33.0°$.

Analysis—$C_5H_{11}N_3O_2$. Calculated, N 26.07; found, N 25.9

DL-α-*Amino-γ-carbamidobutyric Acid*—This was made from DL-α,γ-diaminobutyric acid dihydrochloride obtained from DL-glutamic acid (5). The compound was slightly sweet and melted and frothed vigorously at 200–201° without darkening.

Analysis—$C_5H_{11}N_3O_2$. Calculated, N 26.07; found, N 25.5

A *new synthesis of citrulline* was accomplished by adding 5 gm. of moist nitrourea (6) to the copper complex obtained from 5.0 gm. of DL-ornithine monosulfate in 30 ml. of water, with enough sodium hydroxide solution (dry sodium carbonate replaced the hydroxide in one experiment) to make

---

[4] Prepared by Adamson's method (4) from L-glutamic acid, L-α,γ-diaminobutyric acid dihydrochloride (37.2 per cent Cl calculated, 37.3 per cent Cl found) decomposed at 218–220° on rapid heating and gave $[\alpha]_{546}^{26} = +17.6°$ ($c = 8.04$ in water).

the mixture just alkaline to phenolphthalein.[5] After 4 days standing at room temperature, 2.71 gm. of citrulline copper were filtered off and washed with water and with ethanol. 1 gm. of nitrourea added to the aqueous filtrate gave an additional 0.41 gm. of citrulline copper after standing several days longer. The total yield of copper complex was thus 55 per cent, somewhat lower than the usual yields by the sealed tube method. The crude DL-citrulline from different runs melted variously at 216–217° to 219–220° (decomposition). The substance was recrystallized for analysis.

Analysis—$C_6H_{13}N_3O_3$. Calculated, N 23.98; found, N 23.2

ω-*Phenylcarbamyl Derivatives*—These were prepared from 0.005 to 0.06 mole lots of the copper complexes by agitating the copper derivatives in mildly alkaline aqueous solution with phenyl isocyanate (10 per cent excess) at ice bath temperature. The blue complexes precipitated and were filtered off, washed with water, and were then either washed at once with ethanol or dried and extracted with benzene to remove small amounts of carbanilide. The complexes were most easily decomposed by suspending them in a known amount of dilute hydrochloric acid for the treatment with hydrogen sulfide; addition of an equivalent amount of dilute alkali to the copper-free filtrates precipitated the slightly soluble phenylcarbamyl derivatives.

δ-*Phenylcarbamyl*-DL-*ornithine*—This compound, obtained in 67 per cent yield from the copper salt, gave thin undulant ribbon-like platelets which melted and decomposed sharply at 243.5°. The ninhydrin reaction was strongly positive. The yields of the copper salt itself were 64 to 85 per cent, and when reprecipitated in the usual way melted at 266–267° (decomposition).

Analysis—$C_{12}H_{17}N_3O_3$. Calculated, N 16.72; found, N 16.4
$(C_{12}H_{16}N_3O_3)_2Cu$. Calculated, Cu 11.27; found, Cu 11.26

ϵ-*Phenylcarbamyl*-DL-*lysine*—This substance was isolated from the copper salt (91 per cent from lysine dihydrochloride) in a yield of 68 per cent, with small additional amounts recoverable in the mother liquors by precipitation with cupric acetate solution. On recrystallization from hot water, the phenylcarbamyllysine gave birefringent square or rectangular platelets which, dried over phosphorus pentoxide, melted at 261° to a viscous bubbly mass.

Analysis—$C_{13}H_{19}N_3O_3$. Calculated, N 15.84; found, N 15.7

---

[5] In testing dark blue solutions of copper complexes the phenolphthalein was spotted on a filter paper and a droplet of the unknown placed in juxtaposition. Diffusion of the blue solution into the reagent spot then made detection of faint alkalinity easy.

ε-*Phenylcarbamyl*-L-*lysine*—This was obtained in similar fashion in 63 per cent over-all yield from L-lysine dihydrochloride. The substance crystallized from hot water in rectangular platelets which melted at 258° with decomposition. $[\alpha]_D^{26} = +15.6°$ ($c = 0.20$ M in 2.0 M hydrochloric acid).

Analysis—$C_{13}H_{19}N_3O_3$. Calculated, N 15.84; found, N 15.4

L-α-*Amino*-γ-*phenylcarbamidobutyric Acid*—The copper complex (87 per cent yield from α,γ-diaminobutyric acid dihydrochloride) gave 81 per cent of the phenylcarbamyl amino acid. The substance was slightly soluble in 50 per cent ethanol and in water, from which it crystallized in radiating needles or laths, melting at 233° with gas evolution. $[\alpha]_D^{28} = +13.5°$ ($c = 0.20$ M in 2.0 M hydrochloric acid).

Analysis—$C_{11}H_{15}N_3O_3$. Calculated, N 17.70; found, N 17.7

Benzoyl, p-Nitrobenzoyl, and Phenylsulfonyl Derivatives—These were prepared by direct application of Schotten-Baumann procedures to the copper complexes. The acid chloride in slight excess was added in portions to the refrigerated aqueous solution with vigorous agitation. *p*-Nitrobenzoyl chloride was dissolved in ether; the other halides were added as the substances. Sodium hydroxide, 1, 2, or 15 M, was used for maintaining alkaline reactions.[5] The acylation products precipitated as their copper derivatives and could be readily filtered off, washed, and decomposed. The copper-free filtrates were concentrated to dryness *in vacuo* and the residues recrystallized from hot water, or, less efficiently, from 50 per cent aqueous ethanol.

δ-*Benzoyl*-DL-*ornithine*—This was obtained in an average over-all yield of 72 per cent by the above procedure. When sodium bicarbonate in equivalent amounts was substituted for the sodium hydroxide, the yield of δ-benzoylornithine copper was only 45 per cent, whereas yields with sodium hydroxide approached the theoretical. δ-Benzoyl-DL-ornithine prepared by the copper method, δ-benzoyl-DL-ornithine prepared by acid hydrolysis of DL-ornithuric acid (7), and a mixture of the two, all melted and decomposed simultaneously at 243–248° when heated in one bath.

Analysis—$C_{12}H_{16}N_2O_3$. Calculated, N 11.86; found, N 11.9

The regenerated copper complex melted at 264–265° with decomposition.

Analysis—$(C_{12}H_{15}N_2O_3)_2Cu$. Calculated, Cu 11.90; found, Cu 12.13

ε-*Benzoyl*-DL-*lysine*[4]—This was obtained in over-all yields of 80 to 85 per cent and recrystallized from hot water in thin birefringent rectangular

---

[4] ε-Benzoyl-L-lysine has been prepared in 120 gm. batches by slight modifications of the method previously described (8) for isolating L-lysine from protein hydrolysates.

or elongated and striated hexagonal platelets, melting at 267–279° with decomposition. The melting point was also 267–269° when the compound was mixed with ε-benzoyl-DL-lysine (m.p. 268–270°) prepared by amination (3) of DL-α-bromo-ε-benzoylaminocaproic acid.

Analysis—$C_{13}H_{18}N_2O_3$. Calculated, N 11.20; found, N 11.0

The copper complex, reprecipitated by cupric acetate, melted at 274° with decomposition after being dried over sulfuric acid.

Analysis—$(C_{13}H_{17}N_2O_3)_2Cu$. Calculated, Cu 11.31; found, Cu 11.35

L-α-*Amino-γ-benzoylaminobutyric Acid*—This was obtained in 71 per cent over-all yield from 0.04 mole batches of L-α,γ-diaminobutyric acid dihydrochloride. The substance recrystallized from water in leaflets with pearly luster, m.p. 241° with decomposition, which were soluble in dilute acid and in dilute alkali, but only slightly soluble in absolute ethanol. $[\alpha]_D^{25} = +17.3°$ ($c = 0.20$ M in 2.0 M hydrochloric acid).

Analysis—$C_{11}H_{14}N_2O_3$. Calculated, N 12.61; found, N 12.4

The copper complex gave rosettes of minute bladed needles which decomposed suddenly at 263° after darkening slowly above 225°.

Analysis—$(C_{11}H_{13}N_2O_3)_2Cu$. Calculated, Cu 12.56; found, Cu 12.54

ε-*p-Nitrobenzoyl-DL-lysine*—This was obtained in a single experiment in 74 per cent over-all yield from DL-lysine dihydrochloride. Twice recrystallized the acylated compound gave minute light yellow balls of radiating needles which melted at 242.5–243° with decomposition.

Analysis—$C_{13}H_{17}N_3O_5$. Calculated, amino N 4.74; found, amino N 4.8

ε-*Phenylsulfonyl-DL-lysine*—This was obtained in 78 per cent over-all yields when the reaction bottle was vigorously shaken manually during acylation, with the addition of a few short lengths of glass rod to aid dispersion of the phenylsulfonyl chloride. The ε-phenylsulfonyllysine recrystallized from water in slowly formed aggregates of small rhombic or hexagonal scales, m.p. 216–218° with decomposition. Reprecipitated ε-phenylsulfonyl-DL-lysine copper melted at 243° (decomposition).

Analysis—$C_{12}H_{18}N_2O_4S$. Calculated. N 9.79, S 11.20
Found. " 9.6, " 11.19
$(C_{12}H_{17}N_2O_4S)_2Cu$.[7] Calculated, Cu 10.02; found, Cu 9.92

ε-*Phenylsulfonyl-L-lysine*—This was obtained in 56 per cent over-all yield when the reaction mixture was mechanically stirred during acylation,

---

[7] The corresponding copper derivative of phenylsulfonyl-L-lysine has been formulated (9) as $(C_{12}H_{18}N_2O_4S)_2Cu$.

a procedure obviously not as efficient as the shaking mentioned above. The substance crystallized from water in aggregates of birefringent microscopic blades which melted at 219–221° with gas evolution but no darkening. $[\alpha]_D^{26} = +15.2°$; $[\alpha]_{546}^{26} = +18.0°$ ($c = 0.20$ M in 2.0 M hydrochloric acid). The reprecipitated ε-phenylsulfonyl-L-lysine copper turned dark above 205° and melted at 238° with vigorous decomposition.

Analysis—$C_{12}H_{18}N_2O_4S$. Calculated, N 9.79; found, N 9.8
(C_{12}H_{17}N_2O_4S)_2Cu. Calculated, N 8.83; found, N 8.7

δ-*Phenylsulfonyl*-DL-*ornithine*—This was prepared in 63 per cent yield of the twice recrystallized substance from the copper complex, and the latter was obtained in 76 to 82 per cent yields from ornithine monosulfate. δ-Phenylsulfonylornithine gave brilliant thin leaflets, melting and decomposing at 249° after drying over phosphorus pentoxide.

Analysis—$C_{11}H_{16}N_2O_4S$. Calculated, N 10.29; found, 10.3

L-α-*Amino*-γ-*phenylsulfonylaminobutyric Acid*—This was obtained through the copper complex (72 per cent yield) in 48 per cent over-all yield from L-α,γ-diaminobutyric acid dihydrochloride. Recrystallized from water, the sulfonyl derivative gave rosettes of silky needles, melting at 230–231° (decomposition). $[\alpha]_D^{32} = +22.7°$; $[\alpha]_{546}^{32} = +24.3°$ ($c = 0.20$ M in 2.0 M hydrochloric acid).

Analysis—$C_{10}H_{14}N_2O_4S$. Calculated, N 10.85; found, N 11.3

ω-*Guanyl Derivatives*—Guanyl derivatives of lysine and ornithine were prepared by allowing O-methylisouronium chloride or S-alkylisothiouronium salts[8] to react with the amino acid-copper complexes. The use of the former guanylating agent presented no special difficulties, but S-alkylisothiouronium salts gave pale grayish yellow to grayish green precipitates (probably copper mercaptides) during the course of the reaction, and lower yields of the desired products. Half a dozen variations of the experimental conditions (amount and rate of alkali addition, temperature, length of time allowed for reaction, agitation, and addition of mercuric oxide) failed to eliminate the unwanted precipitates, or to increase yields substantially. Only typical experiments are described below.

DL-*Arginine*[9]—DL-Ornithine monohydrochloride (10.1 gm.) was converted to its copper complex and the resulting solution (75 ml.) cooled and treated with 6.75 gm. of O-methylisouronium chloride. 32 ml. of 2

[8] *S*-Ethylisothiouronium bromide, *S*-(*n*-butyl)isothiouronium bromide, and *S*-methylisothiouronium sulfate were used in different experiments.

[9] Turba and Schuster (10) have recently described the conversion of 585 mg. of synthetic ornithine copper nitrate into arginine flavianate in 94.5 per cent yield by a method essentially similar to that given here.

M sodium hydroxide were then added and the whole allowed to stand at 25–30°. After 5 days, the blue mixture was acidified with concentrated hydrochloric acid, copper removed by hydrogen sulfide, and the combined colorless filtrates and wash water were heated to boiling. When all the excess hydrogen sulfide had been removed, 15.7 gm. of flavianic acid dissolved in 100 ml. of hot water were added with vigorous stirring. DL-Arginine flavianate began to precipitate in a few seconds. The mixture was allowed to cool slowly, with occasional stirring, and was finally refrigerated overnight. The DL-arginine monoflavianate was filtered on a Büchner funnel and washed twice with ice water, and weighed 22.6 gm. after being dried in air at 45–50°. The yield was thus 77 per cent of the ornithine taken. When twice as much (64 ml.) sodium hydroxide was added to the original reaction mixture, the color became green after 12 hours and the final yield fell to 51 per cent. The DL-arginine monoflavianates charred between 255° and 270° without melting. DL-Arginine hydrochloride was prepared from the flavianate by digestion in concentrated hydrochloric acid, filtration, removal of water and hydrochloric acid by distillation *in vacuo*, addition of water, and decolorization with norit, followed by concentration to a thick syrup which was taken up in alcohol and neutralized with pyridine to pH 7, as estimated on a wet indicator paper. Arginine hydrochloride crystallized after refrigeration, and was filtered off and washed well with absolute ethanol and dry ether. Dried over phosphorus pentoxide, the DL-arginine monohydrochloride sintered slowly above 200° and melted at 223° (decomposition). The Sakaguchi reaction was positive.

Analysis—$C_6H_{14}N_4O_2 \cdot HCl$. Calculated. N 26.59, Cl 16.83
                                        Found.   " 26.1, " 17.0

DL-Arginine hydrochloride prepared as described above and L-arginine hydrochloride from gelatin were submitted to the action of partially purified arginase (11) from desiccated fat-free liver powder in strictly parallel experiments. Equal amounts of the two arginine salts in identical volumes were incubated with arginase at 38° for 19 hours. Similar aliquots were then acidified with dilute acetic acid, heated to coagulate proteins, and centrifuged to provide clear aliquots to which glacial acetic acid and alcoholic xanthydrol were added. The weight of washed and oven-dried dixanthydrylurea from the L-arginine was 107.0 mg.; from DL-arginine, 55.8 mg. Dixanthydrylurea from L-arginine hydrolysate, from known urea, from DL-arginine hydrolysate, and a mixture of the latter two, all sintered and melted simultaneously at 256–259° to dark red liquids.

DL-*Homoarginine*—5.55 gm. of *S*-ethylisothiouronium bromide were added to the copper complex from 6.57 gm. of DL-lysine dihydrochloride

in about 35 ml. of water. The mixture was cooled in an ice bath and 16 ml. of 2 M sodium hydroxide were added. The mixture was stirred for 5 hours by drawing air through it with a water pump. After being allowed to stand 2 days at room temperature, the mixture was acidified, copper and hydrogen sulfide were removed, and 9 gm. of flavianic acid were added to the hot filtrate. A red oil precipitated which crystallized on refrigeration.[10] After 2 days, the brilliant orange-red crystals were filtered off and washed successively with cold dilute hydrochloric acid and alcohol. The air-dried flavianate weighed 7.9 gm., a yield of 57 per cent. With twice as much sodium hydroxide in the reaction mixture the yield was 40 per cent. The DL-homoarginine monoflavianate, decomposing at 232°, was converted to the sulfate by stirring 14 gm. of it with 45 ml. of 3 to 4 M sulfuric acid until all the orange-red color was replaced by yellow. The refrigerated mixture was filtered and the flavianic acid washed with dilute sulfuric acid. The filtrate and washings were decolorized with norit and the colorless filtrate neutralized to pH 7 with saturated barium hydroxide solution. The filtrate from barium sulfate was concentrated *in vacuo*, the syrup washed out of the flask with a little hot water, and absolute ethanol was added. A colorless oil precipitated and crystallized overnight in the refrigerator. The mother liquor was poured off and the precipitate dissolved in hot water. Addition of absolute ethanol reprecipitated the sulfate as an oil which crystallized in a few minutes on stirring and scratching. The DL-homoarginine sulfate was dried over phosphorus pentoxide *in vacuo* at 100°, and thereafter intumesced slightly and became translucent at 190–210°; gas evolution became vigorous at 225–250°. The Sakaguchi reaction was positive.

Analysis—$(C_7H_{16}N_4O_2)_2 \cdot H_2SO_4$. Calculated, N 23.62; found, N 23.0

L-*Homoarginine*—O-Methylisouronium chloride (18 gm.) was added to the copper complex prepared from 35 gm. of L-lysine dihydrochloride in 125 ml. of water. 21 ml. of 46 per cent (by weight) sodium hydroxide solution were then added to the cold mixture and the whole was allowed to stand at room temperature for 3 days. From the final copper- and sulfide-free filtrate 34.8 gm. of L-homoarginine monoflavianate were iso-

---

[10] On very rapid cooling or on prolonged standing in the refrigerator, the orange-red crystals of the presumed homoarginine monoflavianate were accompanied by yellow crystals which contained homoarginine, presumably as diflavianate. The nature of the yellow substance was not appreciated during the course of the experiments described, and it was only when the combined crops of yellow crystals from a number of runs were being worked up for the recovery of flavianic acid that the formation of the orange-red monoflavianate from them was observed. The yields stated for homoarginine must therefore be regarded as minimum yields, since they do not include the amounts recovered by working up the yellow crystals.

lated. 42 gm. of the flavianate gave 17.1 gm. of L-homoarginine mono-sulfate, which were dried over sulfuric acid. The yield, calculated as the sesquihydrate (12), was thus 74 per cent from the flavianate and 35 per cent from lysine dihydrochloride. The sulfate was dried over phosphorus pentoxide at 100°. It then gave a positive Sakaguchi reaction, and sintered and melted indefinitely at 183–188°. It gave the following rotations, $[\alpha]_D^{20} = +6.15°$ ($c = 0.20$ M in water), $[\alpha]_D^{26} = +16.9°$ ($c = 0.10$ M in 1.0 M hydrochloric acid), and $[\alpha]_{546}^{26} = +19.4°$ ($c = 0.10$ M in 1.0 M hydrochloric acid).

Analysis—$(C_7H_{16}N_4O_2)_2\cdot H_2SO_4$. Calculated, N 23.62; found, N 22.8

Reprecipitated L-homoarginine monoflavianate, brilliant orange-red platelets, shrank and charred without melting at 247–254°. Benzilidene-L-homoarginine made from the sulfate (12) gave colorless rosettes, melting at 211–212° with decomposition, which retained a benzaldehyde odor even after prolonged washing with ether.

Evidence for Structures Assigned—Evidence of the structure of the compounds synthesized by the copper method may be adduced by using various criteria. In each case when a substance made by the copper method could be directly compared with the same compound made by classical methods, the two were shown to be identical. This was originally done for DL-citrulline (1) and in this paper for δ-benzoyl-DL-ornithine and for ε-benzoyl-DL-lysine. Here it is also shown that DL-arginine obtained by the copper method is hydrolyzed by arginase.

Further evidence that the α-amino group was efficiently masked by copper was obtained by determining the conversion or non-conversion of phenylcarbamyl derivatives to their corresponding phenylhydantoins. Thus 0.7 gm. of δ-phenylcarbamyl-DL-ornithine, m.p. 243.5°, was evaporated to dryness on the steam bath with 15 ml. of concentrated hydrochloric acid. The resultant gum was worked up to give 0.5 gm. of crystals with a melting point of 243–244°, which was undepressed when mixed with some of the original starting material. The failure to form a hydantoin demonstrated that the phenylcarbamyl group could not have been on the α-amino group and must therefore have been on the δ-amino group. Further, δ-phenylcarbamyl-DL-ornithine was benzoylated to give 79 per cent α-benzoyl-δ-phenylcarbamyl-DL-ornithine (clustered blades from ethanol plus water, m.p. 188–189°; calculated for $C_{19}H_{21}N_3O_4$, N 11.82; found, N 11.7) which likewise failed to give any hydantoin. The melting point of my α-benzoyl-δ-phenylcarbamylornithine was undepressed when mixed with the product obtained by the action of phenyl isocyanate on α-benzoyl-DL-ornithine prepared by the method of Boon and Robson (13) and was depressed 20–25° when mixed with the product from the action of phenyl

isocyanate on δ-benzoyl-DL-ornithine prepared by the method of Sörensen *et al.* (7).

Phenyl isocyanate reacted with δ-benzoyl-DL-ornithine prepared by the copper method to give δ-benzoyl-α-phenylcarbamyl-DL-ornithine, m.p. 175° with gas evolution, and this in turn gave 76 per cent of the corresponding phenylhydantoin on evaporation with hydrochloric acid. 5-(γ-Benzoylaminopropyl)-3-phenylhydantoin, flat prisms or needles from ethanol, m.p. 170–170.5°, was negative to the color test for phenylcarbamido groups described below; calculated for $C_{19}H_{19}N_3O_3$, N 13.03; found, N 12.3.

Evidence for the masking of α-amino groups in α,γ-diaminobutyric acid must rest on somewhat different reasoning, since nitrogen analyses will not distinguish between the phenylhydantoins and their isomeric 1-phenylcarbamylpyrrolidones, both of which arise by similar treatment of their corresponding precursors. If α,γ-diaminobutyric acid were to function as a tridentate group, both nitrogen atoms would be masked; if its function were bidentate, two alternatives arise. In the first instance, the two nitrogens would coordinate with cupric ion with consequent masking of both. In the second case, copper would be chelated with oxygen and the α-nitrogen; chelation with oxygen and the γ-nitrogen is unlikely in view of the failure of γ-amino acids to give copper complexes (14). Therefore, if but 1 nitrogen is masked it must be in the α position as ascribed above.

Corroboratory evidence for these ideas was found in the behavior of γ-phenylcarbamido-α-aminobutyric acid when evaporated to dryness with concentrated hydrochloric acid. The product, 1-phenylcarbamyl-3-amino-2-pyrrolidone hydrochloride (73 per cent yield of once recrystallized substance), gave rosettes of needles from water, in which it was much more soluble than in alcohol, m.p. 246–248° (decomposition); calculated for $C_{11}H_{13}N_3O_2 \cdot HCl$, Cl 13.86; found, Cl 13.85. The hydrochloride was converted to the free base, birefringent rectangular platelets, m.p. 105–106°, by neutralization with ammonium hydroxide. In dilute sodium hydroxide, the free base was less than one-eighth as soluble as its parent phenylcarbamidobutyric acid and, unlike the latter, it immediately reduced mercuric oxide formed by adding mercuric chloride to the alkaline solution. Repeated treatment with hot water hydrolyzed the pyrrolidone back to γ-phenylcarbamido-α-aminobutyric acid, m.p. 233–234° (decomposition), undepressed on admixture with the original γ-phenylcarbamido-α-aminobutyric acid. Both the free base and the hydrochloride of 1-phenylcarbamyl-3-amino-2-pyrrolidone gave positive color reactions for phenylcarbamido groups when submitted to the procedure described in the following section. Such reactions are negative in the isomeric phenylhydantoins and are positive here presumably only because of the ease of hydrolysis of the pyrrolidone.

Color Reaction for Compounds Containing Phenylcarbamido Groups—
This reaction was developed and used during the course of this work as a rapid qualitative test. When compounds bearing the $C_6H_5NHCONH—$ group are dissolved in 9 M sulfuric acid and a fragment of potassium

TABLE I

Color Reactions with Potassium Dichromate Given by Phenylcarbamide Derivatives and Related Compounds in 9 M Sulfuric Acid

Substance	Color reaction
δ-Phenylhydantoic acid	Strong red
3-Phenylhydantoin.	Negative
DL-α-Isopropyl-δ-phenylhydantoic acid	Strong red
5-Isopropyl-3-phenylhydantoin	Negative
N-Phenylcarbamyl-L-tyrosine	Red
5-(p-Hydroxybenzyl)-3-phenylhydantoin	Negative
DL-α-n-Propyl-δ-phenylhydantoic acid	Strong red
5-n-Propyl-3-phenylhydantoin	Negative
δ-Benzoyl-α-phenylcarbamyl-DL-ornithine	Strong red
5-(γ-Benzoylaminopropyl)-3-phenylhydantoin	Negative
α-Benzoyl-δ-phenylcarbamyl-DL-ornithine	Strong red
δ-Phenylcarbamyl-DL-ornithine	" "
Di(phenylcarbamyl)-DL-ornithine	" "
L-δ-Phenylcarbamido-α-aminobutyric acid	" "
ε-Phenylcarbamyl-DL-lysine	" "
Phenylurea.	Red-violet
Benzanilide	Negative
"	Red-violet*
Carbanilide	Negative
"	Blue-violet*
Acetanilide.	Negative
"	Weak red*
α-Naphthylurea	Bright green
Biuret	Negative
Ethyl allophanate	"
Hydantoic acid	"
Hydantoin.	"
δ-Phenyl-γ-thiohydantoic acid	"
3-Phenyl-2-thiohydantoin	"
Diphenylguanidine	"

* Color reaction in concentrated sulfuric acid.

dichromate subsequently added, a brilliant, intense red color develops at once. Depending upon the amounts of the materials taken, the color may fade to a dirty brown within a few minutes, or it may persist for an hour or so. Massive dilution with water causes instant decolorization. The reaction is evidently similar to that noted by Tafel (15) to be given

by acetanilide, carbanilide, and the two isomeric N-ethylphenylureas in concentrated sulfuric acid when potassium dichromate is added. Acetanilide and carbanilide give no color in 9 M sulfuric acid, and, even in the concentrated acid, the red color given by acetanilide is much less intense than that given by the phenylcarbamyl derivatives of amino acids. Table I gives the results of tests made on a number of compounds which are sufficiently closely related to the phenylcarbamido compounds to make their behavior worth recording. In making the test, a few mg. of the test substance were dissolved in 1 ml. of the acid, shaken, and then a tiny grain of powdered, recrystallized potassium dichromate was added, and the whole again shaken. No substance which gave a color has been excluded from Table I, although many compounds of unrelated structures which gave negative tests are omitted. Inspection of Table I shows that 3-phenylhydantoins are uniformly negative to the test.

Besides potassium dichromate, potassium permanganate was the only one of the several oxidizing agents tried which gave a color in the test with N-phenylcitrulline. Neither potassium dichromate nor potassium permanganate gave a color when the test substance was dissolved in concentrated hydrochloric acid. Of the compounds thus far tested, any which gives a color in 9 M sulfuric acid also gives a color in concentrated acid, but the converse is not true. Therein lies the possibility of differentiating between the phenylcarbamido acids and certain other phenylureido compounds such as carbanilide.

DISCUSSION

The experimental work recorded above is taken as adequate demonstration of the previously suggested usefulness (1) of the copper method for masking α-amino groups to reagents which attack unprotected amino groups elsewhere in the molecule. Necessity for maintaining a neutral or alkaline medium during reactions is enforced by the instability of the copper complexes in more strongly acid solutions. In acid solutions, the amino nitrogen is coordinated with hydrogen ion instead of cupric ion. The results with the complex formed by α,γ-diaminobutyric acid clearly indicate that the latter functions as a bidentate chelating group and that in the complex the carboxyl oxygen is far more firmly bonded to copper (II) than is the γ-amino nitrogen. It follows, therefore, that the copper (II) complex of α,γ-diaminobutyric acid is not analogous to complexes formed with 1,3-propanediamine.

The copper method permits easy access to amino acid derivatives which may not be readily available otherwise. Syntheses of optically active citrulline and its homologues, and of active homoarginine, illustrate this. The preparation by ordinary methods of a derivative such as ϵ-phenylsul-

fonyllysine may present unexpected difficulties (9) which do not exist for the copper method. The fact that many derivatives of the copper complexes are of low solubility facilitates their isolation, but lack of insolubility in the desired copper derivative does not preclude the use of the method when applied to the preparation of such substances as arginine and homoarginine, which can be readily isolated subsequent to the removal of copper. When insoluble copper complexes are formed, yields tend to be greater when the solubility is less.

Despite its many advantages, there are some conditions under which the copper method cannot be used. Thus far, all attempts to adapt the method to anhydrous media have been unproductive. Failures in the non-aqueous media which have been tried may be ascribed to low solubility of the initial copper complex in the solvent, to reduction of the cupric ion, and to competition by coordinating groups of the solvent for the copper ion which thereby results in unmasking. Even in aqueous media, some reactions cannot be applied. Cyanamide, cold, and allantoin and guanidine at 100° caused reduction of the copper. Thiourea precipitated cupric sulfide. Biuret appeared not to react and methylurea gave citrulline when these were heated to 100° with ornithine copper sulfate in sealed tubes. Various alkyl halides refluxed for long periods with aqueous solutions of the copper complex of lysine and excess cupric carbonate yielded no identifiable N-alkyllysine. In one experiment, picryl chloride and lysine copper chloride gave a precipitate which could not be converted to a crystalline picryllysine. Phenyl isothiocyanate reacted only very slowly and incompletely on agitation with aqueous ornithine copper sulfate at room temperatures.

In a number of instances in which the copper method was successfully employed, parallel experiments without copper demonstrated that different products resulted in the latter case. For example, several benzoylations of DL-ornithine with 1 mole quantities of benzoyl chloride in the absence of copper gave mixtures which presumably contained variable amounts of the two isomeric benzoyl ornithines and ornithuric acid and which melted variously and at much lower temperatures than any of the components. It was thus shown that chelation with copper had a real directive influence on the entering group and that it was impossible to dispense with copper in these reactions.

In the present work, masking of α-amino groups is emphasized, but further application of the method is certainly possible. Preliminary work has suggested that chelation by copper also masks the carboxyl group. Thus, in glutamic acid-copper complex, the γ-carboxyl is left free for reaction while the chelated carboxyl is masked. This phase of the study on masking by copper is being further investigated.

SUMMARY

1. Reactions of the copper complexes of lysine, ornithine, and α,γ-diaminobutyric acid with various reagents have been explored and the copper method has been shown to be a versatile and convenient means for masking the α-amino groups of these amino acids. Some limitations of the method are described.

2. Optically active higher and lower homologues of citrulline have been prepared by the copper method.

3. A new procedure with use of the copper method for preparing citrulline has been presented which allows the synthesis to occur at room temperature.

4. DL-Arginine has been regenerated from ornithine by a copper method and the reaction has been studied to find favorable conditions.

5. L-Homoarginine has been prepared by direct methods from L-lysine copper. DL-Homoarginine has likewise been prepared by simpler procedures and in better yields than in previously published methods.

6. Evidence has been offered for the structures assigned to products obtained by reaction with the copper complexes of the amino acids studied.

7. It has been shown that in α,γ-diaminobutyric acid-copper complex, the copper is attached to a bidentate group containing the α-amino nitrogen and the carboxyl oxygen.

8. It has been suggested that the copper method may be useful for masking carboxyl groups as well as α-amino groups.

9. A color reaction for compounds bearing phenylcarbamido groups has been described.

BIBLIOGRAPHY

1. Kurtz, A. C., *J. Biol. Chem.*, **122**, 477 (1937–38).
2. Duschinsky, R., *Compt. rend. Acad.*, **207**, 735 (1938). Gornall, A. G., and Hunter, A., *Biochem. J.*, **33**, 170 (1939).
3. Eck, J. C., and Marvel, C. S., *J. Biol. Chem.*, **106**, 387 (1934).
4. Adamson, D. W., *J. Chem. Soc.*, 1564 (1939).
5. Bergmann, M., and Zervas, L., *Biochem. Z.*, **203**, 280 (1928).
6. *Org. Syntheses*, coll. **1**, 417 (1941).
7. Sörensen, S. P. L., Höyrup, M., and Andersen, A. C., *Z. physiol. Chem.*, **76**, 44 (1911).
8. Kurtz, A. C., *J. Biol. Chem.*, **140**, 705 (1941).
9. Gurin, S., and Clarke, H. T., *J. Biol. Chem.*, **107**, 395 (1934).
10. Turba, F., and Schuster, K., *Z. physiol. Chem.*, **283**, 27 (1948).
11. Hunter, A., *Biochem. J.*, **33**, 27 (1939).
12. Greenstein, J. P., *J. Org. Chem.*, **2**, 480 (1937–38).
13. Boon, W. R., and Robson, W., *Biochem. J.*, **29**, 2684 (1935).
14. Fischer, E., and Zemplén, G., *Ber. chem. Ges.*, **42**, 4878 (1909).
15. Tafel, J., *Ber. chem. Ges.*, **25**, 412 (1892).

SYNTHESIS OF α- AND β-GLUCOSE-1,6-DIPHOSPHATE*

BY THEODORE POSTERNAK†

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis)

Evidence was recently presented by Leloir *et al.* (2, 3) that phosphoglucomutase requires a coenzyme; its constitution was postulated to be that of glucose-1,6-diphosphate because of the acid lability of a part of its phosphorus and the formation of glucose-6-phosphate by hydrolysis. The coenzyme was isolated from biological material in an estimated purity of 70 per cent. These authors tried to synthesize glucose-1,6-diphosphate by interaction of acetodibromoglucose with silver phosphate; they report that they obtained an enzymatically active mixture containing 0.5 per cent of the coenzyme.

Theoretically glucose-1,6-diphosphate could be synthesized by introduction of a second phosphate group into suitable derivatives of either (*a*) glucose-6-phosphate or (*b*) glucose-1-phosphate. Both procedures were attempted, but the former proved much more successful than the latter.

To avoid obvious complications due to the presence of a free phosphoryl group in the starting material, it was considered necessary to cover it by protecting groups which could be easily removed at a given stage of the synthesis. A suitable starting derivative was found in 1,2,3,4-tetra-acetyl-6-diphenylphosphono-β-D-glucopyranose (I) (4). It is known that in diphenylphosphono derivatives the phenyl groups are readily removed by reductive cleavage with hydrogen in the presence of platinum oxide catalyst (5). When this starting material was treated with hydrobromic acid in acetic acid solution, crystalline α-2,3,4-triacetyl-6-diphenylphosphonoglucosyl bromide[1] (II) was obtained. This intermediate was then submitted to the reactions by which α-glucose-1-phosphate is synthesized from acetobromoglucose (6). By heating in benzene solution with tri-silver phosphate a compound was obtained which may have consisted mainly of a tertiary ester (III) (7). At this stage, it was found necessary

---

to remove the phenyl groups by catalytic hydrogenation. The substance was afterwards treated with hydrochloric acid in methanol in order to remove acetyl groups and to hydrolyze partially the tertiary ester with the liberation of 2 glucose-6-phosphate molecules. The remaining glucose-1,6-diphosphate was finally isolated as the barium salt, which is more soluble in cold than in hot water. This product gave correct analytical values. One-half of its phosphorus was acid-labile. The compound,

$CH_2OPO(OC_6H_5)_2$

(I)

$(C_{24}H_{26}O_{11}P)_3PO_4$
(III)

$Pt+H, HCl, NaOH$

HBr

Ag_3PO_4

$CH_2OPO(OC_6H_5)_2$

(VI)

$Pt+H, NaOH$

$CH_2OPO_3H_2$

(IV)

$CH_2OPO(OC_6H_5)_2$

(II)

$(C_6H_5O)_2OPOAg$

$CH_2OPO(OC_6H_5)_2$

(VII)

$OPO(OC_6H_5)_2$
$Pt+H, NaOH$

$CH_2OPO_3H_2$

(V)
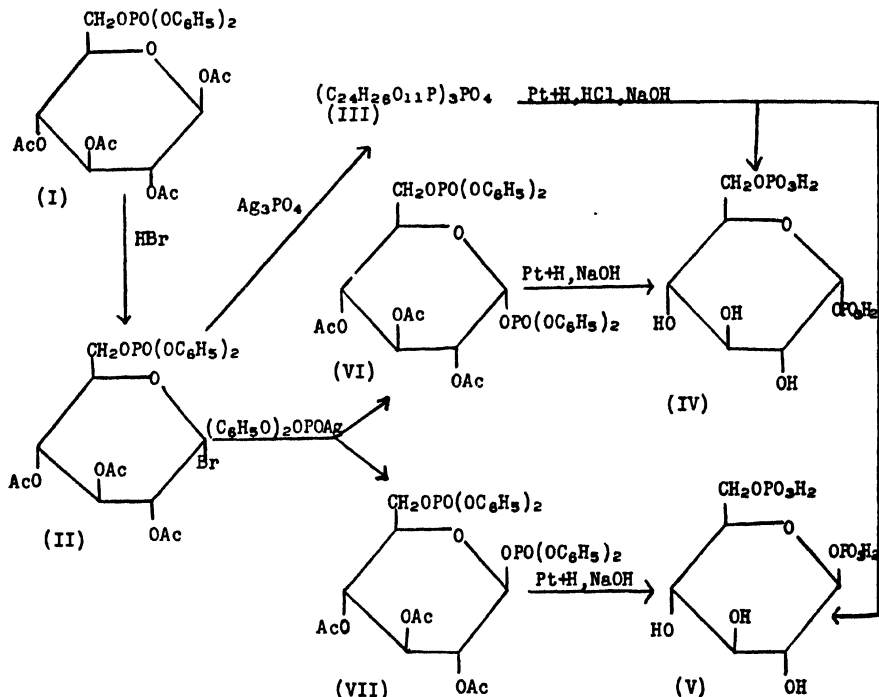
which was non-reducing, had the expected reducing power after hydrolysis. At high dilution, it activated phosphoglucomutase (1).

While the procedure devised by Cori, Colowick, and Cori (6) gave only α-aldohexose-1-phosphates in previous investigations, in the present case several pieces of evidence led to the conclusion that a mixture of α and β forms had been obtained: (1) The rotation ($[\alpha]_D = +12°$ to $+35°$ at pH 8.0)[2] was lower than expected. (2) By incubation (8, 9) with radioactive glucose-1-phosphate and phosphoglucomutase, only a part of the phosphorus of these glucose-1,6-diphosphate samples exchanged with P^{32}. This could be readily explained by the presence of certain amounts

[2] All rotations, unless otherwise stated, are calculated for the free acid.

of the enzymatically inactive β form. (3) A sample was submitted to a mild acid hydrolysis ($5\frac{1}{2}$ hours in normal sulfuric acid at $30°$); 55 per cent of the 1-phosphate groups was removed. The remaining, more resistant glucose-1,6-diphosphate fraction had about twice as high a biochemical activity as the original mixture. This would suggest that the biochemically inactive β form is more easily hydrolyzed.

The final proof of the presence of a mixture of α and β forms was afforded by the separation of the components by fractional crystallization of the brucine salt. The β compound gave a more sparingly soluble salt. In this way, α-glucose-1,6-diphosphate (IV) could be isolated ($[\alpha]_D = +83°$ at pH 8.0). When it was incubated with phosphoglucomutase and radioactive glucose-1-phosphate, the radioactivity was evenly distributed (8, 9). β-Glucose-1,6-diphosphate (V) had, at pH 8.0, $[\alpha]_D = -19°$ and was enzymatically inactive.

Another difference between the α- and the β-glucose-1,6-diphosphates is the rate of hydrolysis of their acid-labile phosphate. At $30°$, in normal sulfuric acid, the first order velocity constant, K $(2.3/t) \log_{10} (A/(A - x))$, for the α-diphosphate is 0.00078; that for the β form is 0.00315 (Table I).[3] Under the same conditions, the hydrolysis constant of α-glucose-1-phosphate has been found to be 0.00336.[4] The 6-phosphate group therefore reduces the rate of hydrolysis, perhaps by "steric hindrance."

A more direct method for the synthesis of α-glucose-1,6-diphosphate was finally discovered. α-2,3,4-Triacetyl-6-diphenylphosphonoglucosyl bromide (II) was treated in benzene solution with silver diphenyl phosphate. The phenyl groups were then removed from the amorphous condensation product (VI) by catalytic hydrogenation in methanol, whereby about 30 per cent of the total phosphorus is split off as inorganic phosphate, and the acetyl groups were removed by alkaline hydrolysis. The glucose-1,6-diphosphate obtained in this way proved to be the pure α form. This method of preparing α-glucose-1,6-diphosphate is simpler and gives a better over-all yield than the trisilver phosphate procedure. If the compound (VI) is hydrogenated in ethanol, 12 per cent only of inorganic phosphate is formed, but the final product contains about 25 per cent β form and 75 per cent α form. These facts can be readily explained by the assumption that the compound (VI) contains some β form (VII) which, when hydrogenated in methanol, is destroyed because of the greater rate of hydrolysis of the β-1,6-diphosphate.

[3] Wolfrom et al. (10) found that the hydrolysis constant of β-glucose-1-phosphate is about 3 times greater than that of the α form.

[4] Cardini et al. (3) observed that the hydrolysis constant of α-glucose-1-phosphate is 4.2 times greater than that of their partially purified coenzyme.

A similar procedure has been successfully applied to the synthesis of α-glucose-1-phosphate.[5]

The condensation of α-acetobromoglucose with two monosilver phosphate derivatives, silver dibenzyl phosphate (10) and "monosilver phosphate" (11), has been described. Inversion was shown to occur with formation of only β-1-phosphate derivatives.[6] Thus there is a remarkable difference between the two reagents mentioned above and silver diphenyl phosphate, which, in the reactions investigated, condenses with α-acetobromo sugars, in the main, without inversion.

A synthesis of glucose-1,6-diphosphate was also attempted by a procedure mentioned above; i.e., by introduction of a phosphate group into

TABLE I

Hydrolysis Rates in 1 N Sulfuric Acid at 30°

The hydrolyses were carried out in stoppered Klett tubes which contained 5 cc. of reaction mixture and were immersed in a bath at 30°. $K = (2.3/t) \log_{10} (A/(A-x))$.

Time	α-Glucose-1,6-diphosphate (20 γ labile P)		β-Glucose-1,6-diphosphate (21.5 γ labile P)		α-Glucose-1-phosphate (24.15 γ labile P)	
min.	per cent P hydrolyzed	$K \times 10^3$	per cent P hydrolyzed	$K \times 10^3$	per cent P hydrolyzed	$K \times 10^3$
45	(3.5)*	(0.588)*	13.6	3.24	13.8	3.29
120	8.9	0.775	30.6	3.04	32.2	3.22
180			43.0	3.13	46.9	3.50
270			57.0	3.17	60.4	3.43
300	21.2	0.791				
870	49.6	0.787				
Average....		0.784		3.15		3.36

* Not included in the average.

the 6 position of glucose-1-phosphate. An enzymatically active material was obtained by the Neuberg phosphorylation procedure (12); that is, by the action of phosphorus oxychloride on glucose-1-phosphate in alkaline solution.[7] However, the yields were small and the purity, after fractionation over the brucine and barium salts, was only 5 per cent.

EXPERIMENTAL

Analytical Methods—For analysis, the substances were dried at 110°, in vacuo, over P_2O_5.[8] Phosphorus was determined by the method of

[5] Unpublished experiments.

[6] β-Glucose-1-phosphate (10) and β-galactose-1-phosphate (11) have been prepared by these methods.

[7] This experiment was carried out with Dr. E. W. Sutherland.

[8] The analyses were in some cases performed on partially dried material. The figures were then corrected for the residual water content.

Fiske and Subbarow (13). The labile phosphate and the reducing power of the glucose-1,6-diphosphate samples were determined after 5 minutes hydrolysis at 100° in normal sulfuric acid. For assay of the reducing power, Nelson's method (14) and Reagent 60 (15) were used, glucose-6-phosphate (G-6-P) being taken as a standard.

α-2,3,4-Triacetyl-6-diphenylphosphonoglucosyl Bromide (II)—5.5 gm. of 1,2,3,4-tetraacetyl-6-diphenylphosphonoglucose (I), prepared according to Lardy and Fischer (4), were suspended in 6 cc. of glacial acetic acid saturated at 0° with dry hydrobromic acid. The mixture was left for 2½ hours at room temperature. If the suspension is frequently shaken, the compound will dissolve in ½ hour. The solution was then diluted with 12 cc. of ice-cold chloroform and shaken with 40 cc. of ice water. The chloroform layer was removed and the water solution was extracted with 6 cc. of chloroform. The combined chloroform extracts were washed several times with small amounts of ice-cold water until the reaction was only faintly acid to Congo red paper. The chloroform solution was dried by shaking for a few minutes with anhydrous calcium chloride. The solution was concentrated by evaporation *in vacuo* at room temperature. Crystals were obtained by slow addition of several volumes of petroleum ether to the concentrated chloroform solution and by letting the mixture stand for a few hours in the cold room. The product was filtered with suction and washed with petroleum ether. For recrystallization the compound was dissolved in 6 cc. of chloroform and petroleum ether was added. The yield was 5.0 gm. and the product was of sufficient purity for subsequent use. The substance crystallizes in long needles and has a double melting point; it melts first at 74–75°, solidifies again, and then melts at 87–88°.

$$[\alpha]_D^{20} = +149° \pm 1.3° \ (c = 1.49 \text{ in chloroform})$$

$C_{24}H_{26}O_{11}PBr$ (601.4). Calculated. P 5.15, Br 13.30

Found. " 5.06, " 13.53

Preparation of α- and β-Glucose-1,6-diphosphates by Condensation of (II) with Silver Phosphate

4.7 gm. of the diphenylbromo compound (II) were dissolved in 14 cc. of dry benzene. 1.2 gm. (0.3 mole) of freshly prepared and finely powdered silver phosphate (7) were suspended in this solution. The mixture was refluxed with exclusion of moisture. Two additional portions of 1.2 gm. of silver phosphate were added after ½ hour and after 1 hour. These three additions of silver phosphate were found necessary for complete removal of bromine from the solution. After 1½ hours, the silver salts were filtered with suction on a sintered glass funnel and washed exhaustively with dry benzene. The combined benzene solutions were evaporated to dryness under reduced pressure (bath temperature 30°).

The last traces of solvent were removed after 5 to 6 hours in a high vac uum at room temperature.

In order to remove the phenyl groups by catalytic hydrogenation, the brittle residue was first dissolved in 30 cc. of dry methanol. This solution, which was filtered to remove traces of silver salts, was shaken at room temperature and at atmospheric pressure with pure hydrogen in the presence of 500 mg. of platinum oxide (Adams' catalyst). After 2 hours, as the uptake became slower, the hydrogenation was interrupted, and 250 mg. more of platinum oxide were added. After 8 hours the theoretical quantity of hydrogen had been absorbed, and the solution was filtered to remove the catalyst and diluted to 100 cc. with methyl alcohol. No inorganic phosphate was formed during the hydrogenation. The mixture contained 333 mg. of organic P which was split by heating in normal sulfuric acid to the extent of 20 per cent after 7 minutes and of 24.5 per cent after 1 hour.

The next step was the partial splitting of the tertiary ester and of some of the acetyl groups. 1.6 cc. of concentrated hydrochloric acid were added to the solution, which was allowed to stand at room temperature. At hourly intervals, 0.1 cc. samples were withdrawn and analyzed for inorganic phosphate. After $11\frac{1}{2}$ hours the inorganic phosphate had risen to 5.6 per cent of the total phosphate (7).

Normal sodium hydroxide was then added to the solution until a permanent pink color was obtained with phenolphthalein. Most of the methyl alcohol was removed by distillation *in vacuo*. The inorganic phosphate was then precipitated by an excess of magnesia mixture prepared with magnesium chloride. After neutralization with acetic acid, the solution was diluted to 200 cc. with water, and a concentrated solution of 3.5 gm. of barium acetate was added. When the mixture was heated to boiling, barium glucose-1,6-diphosphate separated as a flocculent precipitate which was filtered with suction while hot and washed successively with boiling water, alcohol, and ether. An additional amount of material was obtained from the filtrate and wash water by adding 0.25 volume of alcohol; the precipitate was separated by centrifugation and washed first with boiling water, then with alcohol and ether. The total combined yield was 0.7 gm.

The compound was purified by reprecipitation. It was suspended in ice-cold water, and hydrochloric acid was cautiously added until solution was complete. The solution was made weakly alkaline with ammonia and heated to boiling in order to complete the precipitation of the barium salt, which was washed with hot water, alcohol, and ether. The solubility of the barium salt in hot water is about 0.1 per cent and 0.2 to 0.3 per cent in cold water. It has no reducing power, but reducing properties appear after a short acid hydrolysis.

$C_6H_{10}O_{12}P_2Ba_2 \cdot H_2O$ (628.8)

Calculated. C 11.46, H 1.92, total P 9.85, labile P 4.93, G-6-P 41.36

Found. " 11.67, " 1.74, " " 9.90, " " 4.78, " 41.97

For the enzymatic assays, the barium salt was converted to the sodium or potassium salt in the following way: It was suspended in ice-cold water and dissolved with a minimum amount of hydrochloric acid, followed by the addition of the necessary quantity of sodium or potassium sulfate. After neutralization with sodium or potassium hydroxide, the barium sulfate was removed by centrifugation.

Separation of α- and β-Glucose-1,6-diphosphates—This separation can be carried out by a fractional crystallization of the brucine salts. The salt of the β form is less soluble in water; the salt of the α form can be isolated from the mother liquor.

An example of such a separation is the following. The starting material was the barium salt of a mixture which, as sodium salt, had $[\alpha]_D = +35° \pm 1°$ ($c = 0.95$, calculated as free acid in water).

200 mg. (containing 18.0 mg. of P) of the finely powdered barium salt were suspended in 3 cc. of water, and 590 mg. of brucine sulfate were added. The mixture was heated, with constant stirring, for a few minutes at 70° and finally at 100°. It was centrifuged hot and the barium sulfate was exhaustively washed with hot water. Excess barium or sulfate ions must be removed from the liquid by addition of the minimum required amount of brucine sulfate or barium chloride. The solution (volume 10 cc.) was left overnight at 25°. The crystals (200 mg.), consisting mainly of the brucine salt of the β form, were filtered with suction. They were recrystallized repeatedly from 100 times their weight of water. When the quadrangular plates were heated in a capillary tube, they sintered above 170° and decomposed without melting at about 185°. Their solubility in water is about 0.3 per cent at 23° and 0.18 per cent at 4°.

$$[\alpha]_D^{23} = -30.4° \pm 1.6° \ (c = 0.64 \text{ in water})$$
$$C_6H_{14}O_{12}P_2(C_{23}H_{26}O_4N_2)_4. \quad \text{Calculated.} \quad \text{N } 5.84, \text{ P } 3.23$$
$$(1917.9) \qquad\qquad\qquad \text{Found.} \qquad \text{" } 5.65, \text{ " } 3.18$$

The mother liquor of the first crystallization of the brucine salt was evaporated to dryness *in vacuo*. The crystalline residue was washed with 2 cc. of ice-cold water; it contained mainly the salt of the α form, contaminated with some β form. In order to remove the latter, the crystals were dissolved in 5 cc. of water, and the solid, which crystallized at room temperature, was removed. The purity of the soluble fraction can be checked polarimetrically. If its specific rotation is more negative than $-18°$, the fractionation has to be repeated as described above. The mother liquors from the recrystallizations of the β salt contain mixtures of α and β forms which can be separated by the same procedure.

The purified brucine salt of α-glucose-1,6-diphosphate crystallizes out of a concentrated hot aqueous solution in elongated prisms. When heated in a capillary tube, it behaves in the same way as the β salt.

$$[\alpha]_D^{25} = -16.3° \pm 1.3° \ (c = 0.80 \text{ in water})$$

$C_6H_{14}O_{12}P_2(C_{23}H_{26}O_4N_2)_4$. Calculated. N 5.84, P 3.23
(1917.9)   Found.   " 5.67, " 3.20
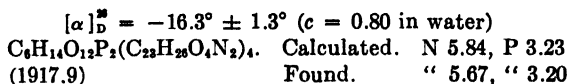
In order to transform the brucine salts into the sodium salts, sodium hydroxide was added to a suspension in water of the brucine salt until a permanent pink color was obtained with phenolphthalein. The solution was then exhaustively extracted with chloroform in order to remove brucine, and the pH was adjusted to 8.0.

For conversion into the barium salts, excess barium acetate was introduced into the solution of the sodium salt. In order to precipitate the barium salt completely, 1 volume of alcohol was added. The precipitate was washed successively with 50 per cent alcohol, absolute alcohol, and ether.

α *Form*—Sodium salt, $[\alpha]_D^{25} = +83° \pm 4° \ (c = 0.229$, calculated as anhydrous free acid in water).
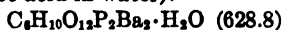
$C_6H_{10}O_{12}P_2Ba_2 \cdot H_2O$ (628.8)
                    Calculated. Total P 9.85, labile P 4.93, G-6-P 41.36
                    Found.   "   " 9.68,   "   " 4.55,   "   42.4

β *Form*—Sodium salt, $[\alpha]_D^{25} = -19° \pm 2° \ (c = 0.373$, calculated as anhydrous free acid in water).
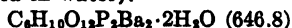
$C_6H_{10}O_{12}P_2Ba_2 \cdot 2H_2O$ (646.8)
          Calculated. C 11.14, H 2.18, total P 9.58, labile P 4.79, G-6-P 40.22
          Found.   " 11.34, " 2.62,   "   " 9.28,   "   " 4.57,   "   38.8

Preparation of α-Glucose-1,6-diphosphate by Condensation of (II) with Silver Diphenyl Phosphate

Silver Diphenyl Phosphate—5 cc. (6.35 gm.) of diphenyl chlorophosphonate (5) were heated on a water bath with 20 cc. of 2.5 N sodium hydroxide until it was completely dissolved. After neutralization to phenolphthalein with concentrated nitric acid, 50 cc. of water and 85 cc. of 10 per cent silver nitrate were added. The solution was heated to boiling and filtered hot to remove the silver chloride. After cooling, the fine needles (yield 4.6 gm.) were filtered with suction and recrystallized from 100 cc. of hot water. The compound was finely powdered and dried at 100°.

α-Glucose-1,6-diphosphate—0.6 gm. of α-2,3,4-triacetyl-6-diphenylphosphonoglucosyl bromide (II) were dissolved in 2 cc. of dry benzene. 0.36 gm. (1 mole) of silver diphenyl phosphate was added and the mixture was refluxed for 15 minutes with exclusion of moisture. 0.2 gm. more of silver diphenyl phosphate was then added and the refluxing continued

for 30 minutes. The silver salts were removed by centrifugation and washed exhaustively with benzene. The solvent was removed from the combined benzene solutions by distillation *in vacuo*. The residue containing the 2,3,4-triacetyl-1,6-di(diphenylphosphono)-glucose (VI) could not be crystallized. For purification, it was dissolved in a small amount of chloroform and reprecipitated with petroleum ether. It was then redissolved in 10 cc. of anhydrous methanol. This solution, which was filtered to remove traces of silver salts, was hydrogenated in the presence of 200 mg. of platinum oxide. The theoretical amount of hydrogen (16 moles) was absorbed in 1 hour. After filtration to remove the catalyst, normal sodium hydroxide was added until a permanent pink color was obtained with phenolphthalein. A gummy precipitate separated which was dissolved by addition of water. The solution, which contained 53 mg. of total phosphorus and 16 mg. (30 per cent) of inorganic phosphorus, was concentrated *in vacuo* in order to remove methanol and cyclohexane. The inorganic phosphate was precipitated with an excess of magnesia mixture prepared from magnesium chloride. The pH of the filtered solution was adjusted to 8.5 with acetic acid. 0.5 gm. of barium acetate was added in concentrated solution and the mixture (20 cc.) was heated to boiling for 2 to 3 minutes. The precipitated salt was centrifuged hot and washed successively with hot water, alcohol, and ether; after being dried by air, the yield was 115 mg., which contained 9.0 per cent total P and 4.2 per cent labile P. Enzymatic assays showed the substance to be fairly pure α-1,6-glucose diphosphate.

For final purification, the compound was transformed into its brucine salt; yield 300 mg. The elongated prisms were recrystallized twice from 10 times their weight of water. Their properties are similar to those indicated above for the brucine salt of α-glucose-1,6-diphosphate obtained by separation of the mixture of the α and β forms.

$$C_6H_{14}O_{12}P_2(C_{23}H_{26}O_4N_2)_4 \text{ (1917.9). Calculated, P 3.23; found, P 3.24}$$
$$[\alpha]_D^{25} = -17.7° \pm 0.8° \; (c = 1.285 \text{ in water})$$
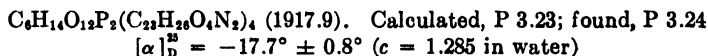
The brucine salt was converted first to the sodium salt.

$$[\alpha]_D^{25} = +80.3° \pm 2.4° \; (c = 0.406, \text{ calculated as anhydrous free acid in water})$$
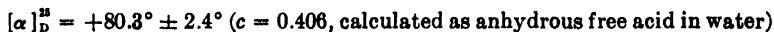
The barium salt was then obtained from a solution of the sodium salt.

$C_6H_{10}O_{12}P_2Ba_2 \cdot H_2O$ (628.8)
Calculated. C 11.46, H 1.92, total P 9.85, labile P 4.93, G-6-P 41.36
Found.   " 11.62, " 1.83, " " 9.88, " " 4.88, " 42.4

When the compound (VI) was hydrogenated in ethanol, only 12 per cent inorganic phosphate was formed and the final yield was about twice as great. However, the isolated glucose-1,6-diphosphate had, as the

sodium salt, a rotation of $[\alpha]_D = +55°$, which indicated that it contained 25 per cent of the β form. The separation of the components could be carried out by fractional crystallization of the brucine salts as described above.

SUMMARY

Two procedures are described for the synthesis of glucose-1,6-diphosphate. The starting material in both procedures is α-2,3,4-triacetyl-6-diphenylphosphonoglucosyl bromide (II) prepared by treatment of 1,2,3,4-tetraacetyl-6-diphenylphosphono-β-D-glucose with hydrobromic acid.

A mixture of α- and β-glucose-1,6-diphosphates was obtained by condensation of the bromo compound (II) with trisilver phosphate, followed by catalytic hydrogenation and partial acid hydrolysis. The α and β forms were separated by fractional crystallization of the brucine salts.

Condensation of the bromo compound (II) with silver diphenyl phosphate, followed by catalytic hydrogenation and alkaline hydrolysis, afforded a better yield of α-glucose-1,6-diphosphate.

BIBLIOGRAPHY

1. Sutherland, E., Posternak, T., and Cori, C. F., *Federation Proc.*, **8**, 258 (1949).
2. Leloir, L. F., Trucco, R. E., Cardini, C. E., Paladini, A., and Caputto, R., *Arch. Biochem.*, **19**, 339 (1948).
3. Cardini, C. E., Paladini, A. C., Caputto, R., Leloir, L. F., and Trucco, R. E., *Arch. Biochem.*, **22**, 87 (1949).
4. Lardy, H. A., and Fischer, H. O. L., *J. Biol. Chem.*, **164**, 513 (1946).
5. Brigl, P., and Müller, H., *Ber. chem. Ges.*, **72**, 2121 (1939).
6. Cori, C. F., Colowick, S. P., and Cori, G. T., *J. Biol. Chem.*, **121**, 465 (1937).
7. Krahl, M. E., and Cori, C. F., in Carter, H. E., Biochemical preparations, New York, **1**, 33 (1949).
8. Sutherland, E. W., Posternak, T. Z., and Cori, C. F., *J. Biol. Chem.*, **179**, 501 (1949).
9. Sutherland, E. W., Cohn, M., Posternak, T., and Cori, C. F., *J. Biol. Chem.*, **180**, 1285 (1949).
10. Wolfrom, M. L., Smith, C. S., Pletcher, D. E., and Brown, A. E., *J. Am. Chem. Soc.*, **64**, 23 (1942).
11. Reithel, F. J., *J. Am. Chem. Soc.*, **67**, 1056 (1945).
12. Neuberg, C., and Pollak, H., *Biochem. Z.*, **23**, 515 (1910); **26**, 514 (1910).
13. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
14. Nelson, N., *J. Biol. Chem.*, **153**, 375 (1944).
15. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100**, 695 (1933).

ACTIVATION OF PHOSPHOGLUCOMUTASE BY METAL-BINDING AGENTS

BY EARL W. SUTHERLAND

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis)

Cysteine, glutathione, and other reducing agents have been used to activate phosphoglucomutase (1, 2), especially crystalline mutase, which was found to be essentially inactive without the addition of cysteine (2). It has been found that cysteine can be replaced by a number of metal-binding agents which approach or equal cysteine in effectiveness. This activation by metal-binding agents has been demonstrated with mutase from skeletal muscle, heart muscle, and yeast and is described in this paper. A summary of these findings has been reported previously (3).

Methods and Preparations

The conversion of glucose-1-phosphate (G-1-P) to glucose-6-phosphate (G-6-P) was measured by the disappearance of labile phosphate; that is, organic phosphate hydrolyzed by normal sulfuric acid during a 5 minute period in a boiling water bath. The inorganic phosphate liberated was then determined by the method of Fiske and Subbarow (4). In some experiments formation of G-6-P was also measured by the appearance of material able to reduce alkaline copper reagents such as the Nelson reagent (5). The usual reaction mixture contained 6×10^{-3} M G-1-P and 1.5×10^{-3} M magnesium sulfate. When cysteine was used, its final molarity was 1.2×10^{-2}. The enzyme preparation was added to a mixture of sufficient volume to permit subsequent withdrawal of aliquots over a period of time. The temperature of incubation was either 30° or 37°.

Crude mutase was prepared by heating water extracts of skeletal or heart muscle 20 minutes at 52° and then removing the denatured protein by filtration. Crystalline mutase was prepared from rabbit muscle as described by Najjar (2). The first crystals were recrystallized once or twice before use. Yeast mutase was found to be enriched in a protein fraction from yeast which represented an intermediate step in the preparation of *Zwischenferment* as described by Negelein and Gerischer (6). Washed and dried brewers' yeast was extracted with 3 volumes of water for 5 hours at 37°, centrifuged, and filtered. Two isoelectric precipitates at pH 4.6 and 8.9 were discarded. The protein fraction precipitating between 0.4 and 0.6 saturated ammonium sulfate was collected and this

fractionation was repeated a second time. The precipitate was dissolved, made 0.4 saturated with ammonium sulfate, and heated for 1 hour at 40°. Denatured protein was discarded and the solution was dialyzed for 19 hours against distilled water.[1] This preparation is very similar to one which has been used for the preparation of the enzyme from aqueous extracts of muscle.

Glucose-1-phosphate was prepared chemically by the method of Cori, Colowick, and Cori (7), or enzymatically with starch, inorganic phosphate, and phosphorylase from potatoes or from rabbit muscle.[2]

<center>EXPERIMENTAL</center>

When amorphous, zinc-free, or well dialyzed crystalline zinc insulin was added to muscle mutase, either crude or purified, the rate of the enzymatic reaction was increased. This stimulation occurred in the absence of cysteine, required relatively large amounts of insulin, and was non-specific in the sense that other proteins, e.g. crystalline albumin, could be substituted for insulin. By contrast it was found that non-dialyzed zinc insulin inhibited mutase. This inhibition is probably caused by zinc ions, to which the enzyme is quite sensitive (Table I). It suggested moreover that the activation of mutase by proteins and by cysteine represented a metal-binding effect. A number of heavy metal-binding agents were therefore investigated; they differ from cysteine, which is also a heavy metal binder, in having no reducing action.

Histidine was found to be more effective than insulin (Fig. 1), and in larger concentrations (0.04 M) approached cysteine in the degree of activation of mutase. Histamine was less effective than histidine, while carnosine had no activating effect. (Fig. 1).

Diphenylthiocarbazone (dithizone) is a metal-binding agent which is almost insoluble in water, but can be used in carbon tetrachloride solution. When the complete reaction mixture was shaken with carbon tetrachloride containing 0.01 per cent dithizone, a rapid enzymatic reaction took place, while the control, which was shaken with the same amount of carbon tetrachloride containing no dithizone, showed no reaction at all (Fig. 2). 8-Hydroxyquinoline, being more soluble in water, could be added directly to the reaction mixture and approached optimal amounts of cysteine in effectiveness (Fig. 2). Activation by 8-hydroxyquinoline or cysteine could also be demonstrated with mutase from heart muscle and from yeast (Fig. 3). In contrast to the mutase preparations of mammalian tissues that of yeast showed considerable activity in the absence of metal-binding

---

[1] Dr. M. Slein kindly provided a sample at this stage.

[2] The preparations used contained glucose-1,6-diphosphate in amounts sufficient to give maximal activation with respect to this coenzyme.

Effect of Metal Ions on Activity of Yeast Mutase

The reaction mixture consisted of 6×10^{-3} M glucose-1-phosphate and 1.5×10^{-3} M magnesium ions. No metal-binding agents were added.

Metal	Charge	Concentration of metal, final molarity	Inhibition
			per cent
Cobalt . .	++	5×10^{-5}	0
Iron	++	5×10^{-5}	0
Zinc .	++	1×10^{-4}	91
" . .	++	5×10^{-5}	53
Lead . .	++	1×10^{-4}	85
Copper . .	++	1×10^{-4}	100
"	++	5×10^{-6}	33
Mercury	++	5×10^{-5}	97
Silver	+	5×10^{-5}	96
" .	+	5×10^{-6}	32
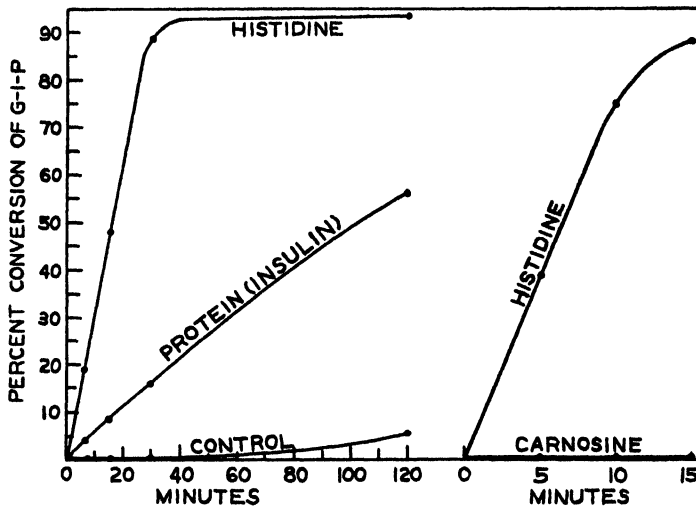
FIG. 1. Effect of histidine, insulin, and carnosine on crystalline phosphoglucomutase. The reaction mixtures contained enzyme, 6×10^{-3} M glucose-1-phosphate, and 1.5×10^{-3} M magnesium ions. The final concentration was 0.024 M for histidine in the first experiment and 0.032 M for histidine and carnosine in the second experiment. Crystalline zinc insulin was dialyzed 18 hours against acidified distilled water, and was then added to give a final concentration of 1.3 mg. per ml.

agents. The inhibition caused by the addition of small amounts of zinc ions was completely overcome by the addition of the metal-binding agents (Fig. 3).

In several experiments with yeast mutase varying amounts of metal ions were added to the reaction mixture, which included 1.5×10^{-3} M magnesium ion but no metal-binding agent. Metal ions which inhibited the yeast enzyme in low concentrations were zinc, lead, copper, mercury, and silver (Table I). Copper and silver ions partially inhibited the spontaneous activity of the yeast mutase at 5×10^{-6} M concentration, but, since other proteins were present as impurities, the enzyme might actually be
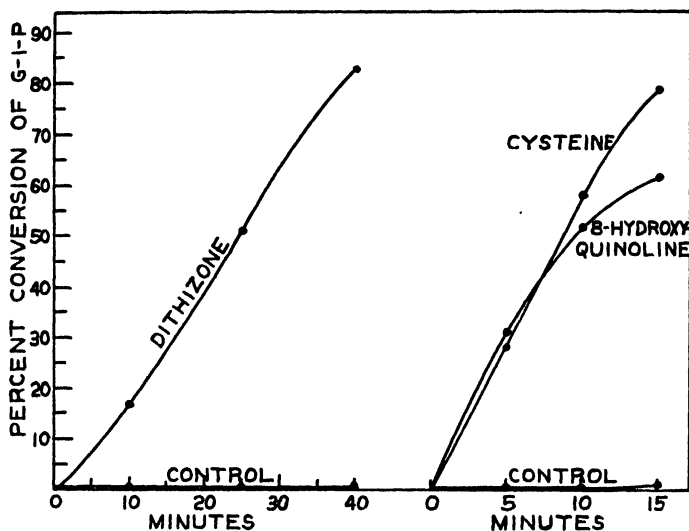
FIG. 2. Effect of dithizone, cysteine, and 8-hydroxyquinoline on crystalline phosphoglucomutase. The reaction mixtures contained enzyme, 6×10^{-3} M glucose-1-phosphate, and 1.5×10^{-3} M magnesium ions. In the first experiment the reaction mixture was shaken with carbon tetrachloride alone in the control and with carbon tetrachloride containing 0.01 per cent dithizone in the other. In the second experiment the final molarity of cysteine was 0.025 and that of the 8-hydroxyquinoline 0.002.

considerably more sensitive to these metals. Crystalline mutase could not be used in such experiments because it was inactive without the addition of metal-binding agents. This is presumably due to the presence of traces of heavy metal. The inhibition of yeast mutase by metal ions was reversible under the conditions tested; i.e., after complete inhibition of the mutase reaction was obtained with metal ions, addition of metal-binding agents restored the rate to that of the original uninhibited reaction.

Since the marked sensitivity to metals suggested involvement of sulfhydryl groups, the effect of iodoacetate was investigated. When 1×10^{-3}

M iodoacetate was added directly to the reaction mixture, no inhibition was noted. However, when the yeast enzyme was preincubated with 2×10^{-3} M iodoacetate for 10 minutes at room temperature before addition to the reaction mixture, an inhibition was observed which amounted to about 50 per cent.
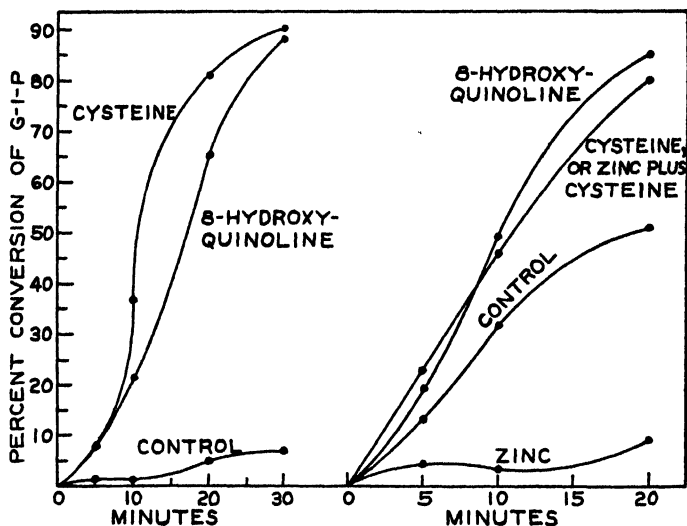
FIG. 3. Effect of metal-binding agents on phosphoglucomutase from heart (first experiment) or yeast (second experiment). The reaction mixtures contained enzyme, 6×10^{-3} M glucose-1-phosphate, and 1.5×10^{-3} M magnesium ions. In both experiments the final molarity of cysteine was 0.02 and that of 8-hydroxyquinoline 0.003. When added, zinc ions were present in a final concentration of 5×10^{-5} M.

DISCUSSION

Apart from metal-binding agents, phosphoglucomutase requires glucose-1,6-diphosphate for its activity (see the following paper (8)). The experiments reported in this paper were carried out in the presence of sufficient glucose-1,6-diphosphate to saturate the enzyme with respect to this cofactor. In the absence of metal-binding agents, 1,6-diphosphate, even in a final concentration of 2×10^{-3} M, failed to activate crystalline mutase.

Cysteine, which is a reducing agent as well as a metal-binding agent, was found to be slightly more effective than other metal-binding agents. It is known from work with other enzymes (phosphorylase, glyceraldehyde phosphate dehydrogenase) that the "reducing action" of cysteine as measured by the increase in enzyme activity is not instantaneous but requires 5 or more minutes for completion. By contrast phosphogluco-

mutase shows a rapid decrease in activity when it is exposed to the reducing action of cysteine. In order to obtain maximal activation of mutase with cysteine, the latter must not be in contact with the enzyme prior to the start of the reaction (2). It therefore seems probable that cysteine activates mutase merely in its capacity as a metal binder.

Since proteins are metal-binding agents, crude mutase preparations in general show less need of metal-binding agents than do purified preparations. With a relatively crude yeast fraction, addition of metal-binding agents increased the activity by about 50 per cent. Addition of small amounts of heavy metal ions magnified the effect of metal-binding agents. Much stronger activation by metal-binding agents was observed with crude mutase preparations from heart or skeletal muscle. Such differences may in part be accounted for by the capacity of the contaminating proteins to bind metal ions. That muscle extract contains a protective protein fraction has been observed previously (9): The crystalline enzyme from muscle is apparently extremely sensitive to traces of heavy metal, since it is completely inactive in the absence of metal-binding agents.

SUMMARY

Phosphoglucomutase from skeletal muscle, heart, or yeast can be activated by metal-binding agents. Various proteins (zinc-free insulin, serum albumin) exert such an effect which is not, however, maximal. Zinc insulin causes inhibition, since the enzyme is quite sensitive to zinc ions (as well as to copper, mercury, and silver ions). Histidine, diphenylthiocarbazone, and 8-hydroxyquinoline were found to activate the enzyme nearly as effectively as cysteine. The activation by these metal-binding agents is distinct from the activation by glucose-1,6-diphosphate.

BIBLIOGRAPHY

1. Lehmann, H., *Biochem. J.*, **33**, 1241 (1939).
2. Najjar, V. A., *J. Biol. Chem.*, **175**, 281 (1948).
3. Sutherland, E., Posternak, T., and Cori, C. F., *Federation Proc.*, **8**, 258 (1949).
4. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
5. Nelson, N., *J. Biol. Chem.*, **153**, 375 (1944).
6. Negelein, E., and Gerischer, W., *Biochem. Z.*, **284**, 289 (1936).
7. Cori, C. F., Colowick, S. P., and Cori, G. T., *J. Biol. Chem.*, **121**, 465 (1937).
8. Sutherland, E. W., Cohn, M., Posternak, T., and Cori, C. F., *J. Biol. Chem.*, **180**, 1285 (1949).
9. Cori, G. T., Colowick, S. P., and Cori, C. F., *J. Biol. Chem.*, **124**, 543 (1938).

THE MECHANISM OF THE PHOSPHOGLUCOMUTASE REACTION

By EARL W. SUTHERLAND, MILDRED COHN, THEODORE POSTERNAK, AND CARL F. CORI

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis)

Although many samples of glucose-1-phosphate had been prepared in this laboratory in the last 12 years, it was not until recently that an unusual observation was made with one of them. This sample was found by Dr. Najjar to be inactive as substrate for phosphoglucomutase, while it retained its full activity as substrate for phosphorylase. In pursuit of this observation other preparations of glucose-1-phosphate, both natural and synthetic, were tested with phosphoglucomutase. It was found that these preparations varied in the rate of conversion to glucose-6-phosphate from maximal activity to a barely perceptible rate of enzymatic conversion. Addition of an active to an inactive sample of glucose-1-phosphate resulted in a rapid disappearance of both, suggesting that the active sample contained a hitherto unrecognized activator for the phosphoglucomutase system.

It would undoubtedly have been a difficult problem to establish the nature of this activator, but this was brilliantly solved by Leloir and coworkers (1, 2) when they suggested that the activator might be glucose-1,6-diphosphate.

By way of confirmation, and because of the interest of this laboratory in both the enzyme and the 1-phospho sugars, it was thought appropriate to attempt a synthesis of this compound. Methods for the preparation of α-glucose-1,6-diphosphate are described in a preceding paper (3). On addition of catalytic amounts of this compound to inactive samples of glucose-1-phosphate, the rate of conversion reached that observed with active preparations of glucose-1-phosphate alone.

The mode of participation of glucose-1,6-diphosphate in the transformation of glucose-1-phosphate to glucose-6-phosphate was studied by means of isotopic tracers. P^{32} was used to label the phosphate group and C^{14} to label the glucose moiety of glucose-1-phosphate. On incubation with crystalline phosphoglucomutase and non-isotopic glucose-1,6-diphosphate, equilibration of the phosphorus and carbon between the two hexose monophosphates and the diphosphate was observed.

Activation of Phosphoglucomutase Reaction

Effect of Different Samples of Glucose-1-phosphate—The test system for phosphoglucomutase activity has been described in detail previously (4). Metal-binding agents and Mg ions are necessary for full enzyme activity, but, even when they are present in optimal amounts, one still observes that various samples of glucose-1-phosphate, either synthetic or natural,
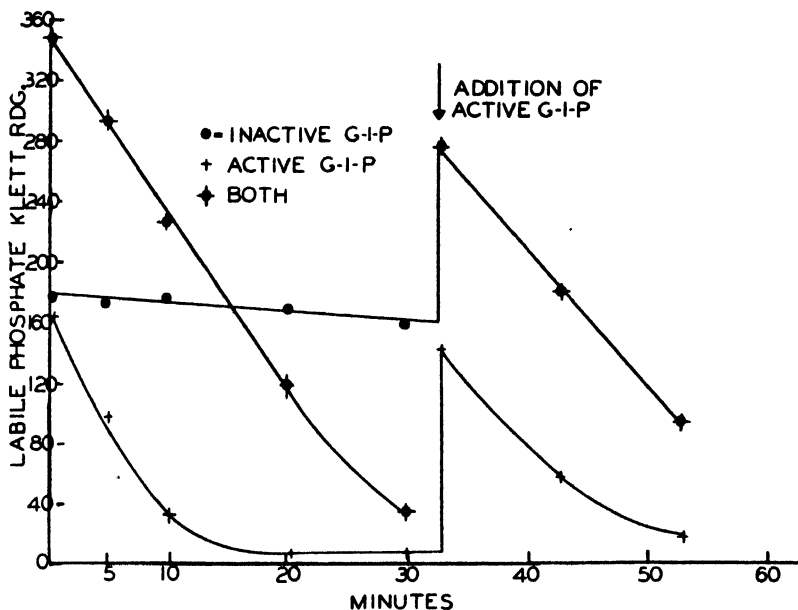
FIG. 1. Rate of conversion of inactive glucose-1-phosphate to glucose-6-phosphate with and without the additon of active glucose-1-phosphate in a phosphoglucomutase (yeast) reaction mixture. The active sample was added to the inactive sample at the start of the reaction in the top curve, after 33 minutes of incubation in the center curve, and at 33 minutes to the active sample in the bottom curve. The ordinate scale represents Klett-Summerson colorimeter readings.

differ greatly in their rate of conversion to glucose-6-phosphate. This applies to relatively crude enzyme preparations from yeast (4) as well as to crystalline phosphoglucomutase from rabbit muscle (5). Figs. 1 and 2 show typical experiments with these two enzyme preparations. In Fig. 1 colorimeter readings (Klett-Summerson) rather than per cent conversion of glucose-1- to glucose-6-phosphate are plotted against time in order to permit direct comparison of rates of conversion in the presence of varying amounts of substrate. It may be seen that the addition of active to inactive glucose-1-phosphate allows the latter to be attacked by

the enzyme. Furthermore, the addition of the active to the inactive sample, either at the start of the reaction or after 33 minutes of incubation, increased the rate of conversion of the mixture to that of the active sample alone. The same result is obtained when a catalytic amount of glucose-1,6-diphosphate is added to inactive glucose-1-phosphate, as shown in the next section.
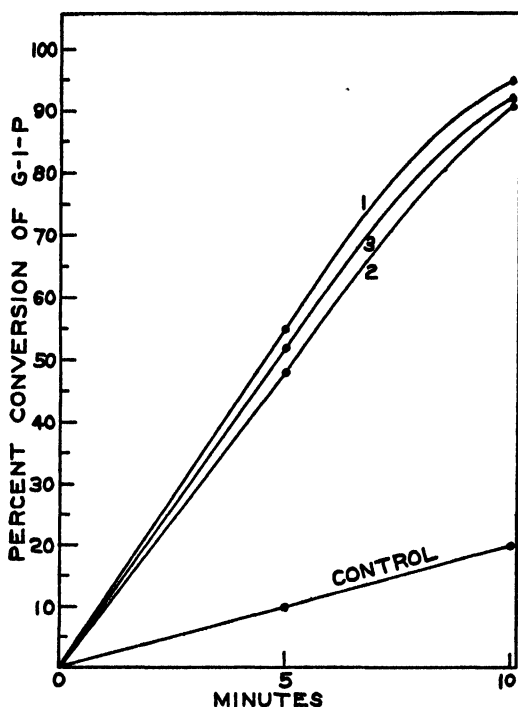
FIG. 2. Rate of conversion of inactive glucose-1-phosphate to glucose-6-phosphate with and without the addition of glucose-1,6-diphosphate in a phosphoglucomutase (muscle) reaction mixture. The concentration of glucose-1,6-diphosphate was 1.7×10^{-6} M in Curve 1 and 8×10^{-6} M in Curve 2. Curve 3 shows the rate of conversion of the active sample of glucose-1-phosphate used in Fig. 1.

Activation by α-Glucose-1,6-diphosphate—In Fig. 2 the rates of conversion of maximally active glucose-1-phosphate (Curve 3) and of an inactive sample with added glucose-1,6-diphosphate (Curves 1 and 2) are all shown to be nearly the same. Considering that a concentration of about 2×10^{-6} M gave maximal activation, the enzyme must have a high affinity for glucose-1,6-diphosphate. This is borne out in the experiment in Fig. 3, which shows that half maximal activity was observed with a con-

centration of α-glucose-1,6-diphosphate of the order of 5×10^{-7} mole per liter.

A somewhat higher value reported previously (6) was due to the fact that the synthetic preparation of the diphosphate then used was a mixture of the α and β forms. It has since been found that β-glucose-1,6-diphosphate does not have an activating effect on the enzyme.

Assay for Glucose-1,6-diphosphate—By means of the enzyme velocity-coenzyme concentration curve, expressed by $K = ((V - v)/v) \times c$, where
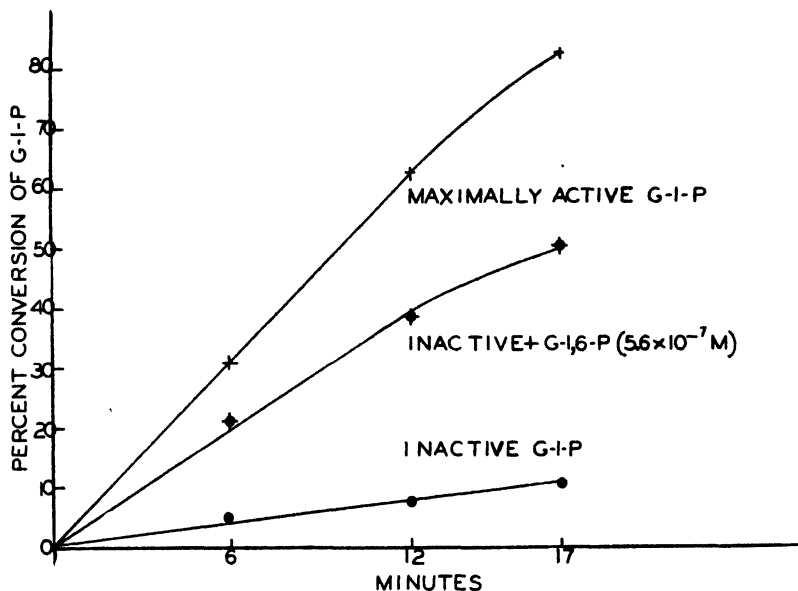
FIG. 3. Effect of a submaximal amount of glucose-1,6-diphosphate on the rate of conversion of inactive glucose-1-phosphate to glucose-6-phosphate in a phosphoglucomutase (muscle) reaction mixture.

K corresponds to that concentration of coenzyme which gives half maximal velocity (in our case 5×10^{-7} M), one can determine the amount of glucose-1,6-diphosphate in an unknown sample. Such determinations were of particular interest in the case of various preparations of glucose-1-phosphate. It was found that synthetic glucose-1-phosphate,[1] depending on the method of preparation and purification, contained from practically 0 to 0.03 per cent of glucose-1,6-diphosphate. In the case of enzymatically prepared glucose-1-phosphate some samples contained as much as 0.1 per cent glucose-1,6-diphosphate or 3 times the amount necessary for maximal

[1] Prepared by the method of Cori, Colowick, and Cori (7) as well as by methods referred to in a preceding paper (3).

activation of the enzyme (in the usual reaction mixture containing 6×10^{-3} M glucose-1-phosphate). The factors which lead to the formation of glucose-1,6-diphosphate in a system consisting of polysaccharide, inorganic phosphate, and phosphorylase are being investigated.

Exchange Experiments

Preparation of Glucose-1-phosphate (P^{32})—This was prepared by exchange of crystalline non-radioactive glucose-1-phosphate with radioactive inorganic phosphate (5 γ of P) in the presence of sucrose phosphorylase (8). Sucrose phosphorylase was obtained from dried *Pseudomonas saccharophila*[2] by the method of Doudoroff (9). At the end of the reaction, the radioactive inorganic phosphate was diluted with non-radioactive inorganic phosphate and removed by precipitation with magnesia mixture; this procedure was repeated twice, the first time 8 mg. of inorganic P were added, the second time, 6 mg. The glucose-1-phosphate (P^{32}) was crystallized and recrystallized twice as the potassium salt. To the crystalline product were added 500 mg. of non-radioactive potassium salt of glucose-1-phosphate and the mixture was washed out with non-radioactive inorganic phosphate (16 mg. of P). The inorganic phosphate was removed with magnesia mixture and the potassium salt of glucose-1-phosphate was recrystallized twice.

Preparation of Glucose-1-phosphate (C^{14})—Glucose-1-phosphate containing radioactive carbon was prepared from radioactive starch[3] (2 mg.) incubated with inorganic phosphate and crystalline muscle phosphorylase. At the end of 1 hour, salivary amylase was added and the reaction mixture was reincubated for a half hour and then heated for 10 minutes to destroy enzyme activity. The glucose-1-phosphate (C^{14}) formed was isolated in crystalline form after addition of 150 mg. of non-radioactive dipotassium glucose-1-phosphate as carrier. 10 mg. of non-radioactive starch were then added and the amylase digestion was repeated. The glucose-1-phosphate was again crystallized and recrystallized as the dipotassium salt from alcohol. Any precipitate appearing at pH 4 in alcohol was removed before crystallization of the glucose-1-phosphate.

Separation of Phosphate Esters—The method of separation is based on the fact that the barium salt of the diphosphate ester precipitates almost quantitatively from hot, slightly alkaline solution, while glucose-1-phosphate and glucose-6-phosphate remain in solution.

After incubation with phosphoglucomutase, the reaction mixture was heated at 100° for 10 minutes and the coagulated protein was removed by centrifugation. To precipitate the barium salt of glucose-1,6-diphosphate,

---

[2] Kindly furnished by Dr. W. Z. Hassid of the University of California.
[3] Kindly furnished by Dr. M. Gibbs of the Brookhaven National Laboratory.

a concentrated barium acetate solution was added in slight excess to the supernatant solution and the solution was made alkaline to brom thymol blue with ammonium hydroxide. The precipitation was brought to maximum by heating at 100° for 2 minutes. The precipitate was separated by rapid centrifugation in a heated cup. In order to remove any contamination of the diphosphate ester by radioactive monophosphate esters, the product was washed three times with a boiling solution of an equilibrium mixture of glucose-6-phosphate and glucose-1-phosphate (95:5) containing 0.45 mg. of P per cc. and was subsequently washed three times with boiling water. The washed barium salt of the glucose-1,6-diphosphate was dissolved in ice-cold hydrochloric acid, just acid to Congo red paper; any trace of BaSO₄ which had formed due to the presence of ammonium sulfate in the enzyme preparations was removed by centrifugation. The glucose-1,6-diphosphate was reprecipitated by making the solution alkaline to brom thymol blue with ammonium hydroxide and heating. The precipitate was again centrifuged while hot. To replace Ba^{++} by Na^+, the precipitate was suspended in ice-cold water and ice-cold hydrochloric acid was added dropwise until the precipitate was completely dissolved. A slight excess of 5 per cent Na_2SO_4 solution was added, the BaSO₄ formed was centrifuged in the cold, and the supernatant solution was neutralized with NaOH.

The glucose monophosphate was obtained from the initial solution from which the diphosphate had been removed as the barium salt. Enough alcohol was added to yield a 20 per cent solution. The precipitate formed at this alcohol concentration was discarded, since it contained small amounts of glucose-1,6-diphosphate, and the alcohol concentration was increased to 80 per cent. The precipitate which formed was centrifuged, washed with alcohol, then with ether, and dried *in vacuo*. The precipitate was dissolved in a small amount of hot water and any undissolved material was discarded. The Ba^{++} was removed from solution with Na_2SO_4 as already described for the diphosphate. Aliquots of the solutions of the sodium salts of the monophosphate and diphosphate esters, each of whose phosphorus content had been determined, were evaporated to dryness under an infra-red lamp for measurement of radioactivity.

Measurement of Radioactivity—The radioactivity of all samples was measured with a thin mica window counter. The samples which contained both P^{32} and C^{14} were measured with and without an aluminum filter 4 mils in thickness. This filter absorbed all the carbon radiation and 32 per cent of the phosphorus radiation under the conditions used. The radioactivity of the phosphorus in samples about 1 to 1.5 mg. per sq. cm. was determined directly from measurements with the filter by which only the phosphorus radiation was recorded. The radioactivity of carbon was measured in samples whose thickness (0.1 to 0.2 mg. per sq. cm.) was

activation of the enzyme (in the usual reaction mixture containing 6×10^{-3} M glucose-1-phosphate). The factors which lead to the formation of glucose-1,6-diphosphate in a system consisting of polysaccharide, inorganic phosphate, and phosphorylase are being investigated.

Exchange Experiments

Preparation of Glucose-1-phosphate (P^{32})—This was prepared by exchange of crystalline non-radioactive glucose-1-phosphate with radioactive inorganic phosphate (5 γ of P) in the presence of sucrose phosphorylase (8). Sucrose phosphorylase was obtained from dried *Pseudomonas saccharophila*[2] by the method of Doudoroff (9). At the end of the reaction, the radioactive inorganic phosphate was diluted with non-radioactive inorganic phosphate and removed by precipitation with magnesia mixture; this procedure was repeated twice, the first time 8 mg. of inorganic P were added, the second time, 6 mg. The glucose-1-phosphate (P^{32}) was crystallized and recrystallized twice as the potassium salt. To the crystalline product were added 500 mg. of non-radioactive potassium salt of glucose-1-phosphate and the mixture was washed out with non-radioactive inorganic phosphate (16 mg. of P). The inorganic phosphate was removed with magnesia mixture and the potassium salt of glucose-1-phosphate was recrystallized twice.

Preparation of Glucose-1-phosphate (C^{14})—Glucose-1-phosphate containing radioactive carbon was prepared from radioactive starch[3] (2 mg.) incubated with inorganic phosphate and crystalline muscle phosphorylase. At the end of 1 hour, salivary amylase was added and the reaction mixture was reincubated for a half hour and then heated for 10 minutes to destroy enzyme activity. The glucose-1-phosphate (C^{14}) formed was isolated in crystalline form after addition of 150 mg. of non-radioactive dipotassium glucose-1-phosphate as carrier. 10 mg. of non-radioactive starch were then added and the amylase digestion was repeated. The glucose-1-phosphate was again crystallized and recrystallized as the dipotassium salt from alcohol. Any precipitate appearing at pH 4 in alcohol was removed before crystallization of the glucose-1-phosphate.

Separation of Phosphate Esters—The method of separation is based on the fact that the barium salt of the diphosphate ester precipitates almost quantitatively from hot, slightly alkaline solution, while glucose-1-phosphate and glucose-6-phosphate remain in solution.

After incubation with phosphoglucomutase, the reaction mixture was heated at 100° for 10 minutes and the coagulated protein was removed by centrifugation. To precipitate the barium salt of glucose-1,6-diphosphate,

[2] Kindly furnished by Dr. W. Z. Hassid of the University of California.
[3] Kindly furnished by Dr. M. Gibbs of the Brookhaven National Laboratory.

phate—Two experiments were carried out with glucose-1-phosphate labeled with P^{32} and C^{14}. In the first experiment, the reaction mixture contained the following components in a total volume of 13.5 cc.: 8-hydroxyquinoline, 7 mg.; sodium salt of glucose-1,6-diphosphate, 4.25 mg. of P; potassium salt of glucose-1-phosphate (C^{14}), 0.88 mg. of P; potassium salt of glucose-1-phosphate (P^{32}), 4.30 mg. of P; $MgCl_2$, 0.1 mM; third phosphoglucomutase crystals, 2.2 mg. of protein. Before the addition of the enzyme, a sample was withdrawn for phosphate analysis, labile and total. The reaction mixture was incubated at 30° at pH 7.1 At the end of 8 minutes, a sample was withdrawn for analysis of labile phosphate; equilibrium had been reached in the conversion of glucose-1-phosphate to glucose-6-phosphate, since the amount of labile phosphate remaining corresponded to the amount of labile phosphate in the glucose-1,6-diphosphate initially added plus 5

TABLE I

Equilibration of Glucose Monophosphate (C^{14}, P^{32}) and Glucose-1,6-diphosphate in Phosphoglucomutase Reaction

The sample of glucose-1,6-diphosphate used in this experiment consisted of a mixture of the α and β forms. The results are expressed in counts per minute per mg.

Sample	P^{32}			C^{14}		
	Initial	40 min.	9 hrs.	Initial	40 min.	9 hrs.
Glucose monophosphate	*	870	905	12,500	11,575	11,200
Glucose-1,6-diphosphate	0	430	400	0	4,950	5,560

' This sample was inadvertently lost.

per cent of the total hexose monophosphate P. Half of the remaining solution was withdrawn after 40 minutes for separation of the diphosphate and monophosphates and the determination of their P^{32} and C^{14} content. After 9 hours of incubation, the diphosphate and monophosphate fractions were separated from the remainder of the reaction mixture and their radioactivity measured. As is shown in Table I, both the P^{32} and C^{14} values for the diphosphate ester at 40 minutes and 9 hours were approximately 50 per cent of the values found for the monophosphate esters, instead of the equal values to be expected for complete equilibration. Again, this was due to the fact that the glucose-1,6-diphosphate used in this experiment was a mixture of the α and β forms.

In the second experiment, pure α-glucose-1,6-diphosphate was used. The reaction proceeded under the same conditions as in the first experiment on a somewhat smaller scale, the amount of diphosphate corresponding to 1.5 mg. of P, glucose-1-phosphate (C^{14}) to 0.42 mg. of P, and glucose-1-phosphate (P^{32}) to 2.2 mg. of P. An analysis for labile phosphate after

8 minutes of incubation showed that equilibrium had been established in so far as the interconversion of glucose-1-phosphate and glucose-6-phosphate was concerned. At the end of 2 hours, the diphosphate and monophosphates were separated and their radioactivities determined as given in Table II. In this case, complete equilibration had been achieved between the diphosphate and monophosphates.

TABLE II

Equilibration of Glucose Monophosphate (C^{14}, P^{32}) and 1,6-Diphosphate in Phosphoglucomutase Reaction .

Pure α-glucose-1,6-diphosphate was used in this experiment. The results are expressed in counts per minute per mg.

Sample	P^{32}			C^{14}		
		Final, 2 hrs.			Final, 2 hrs.	
	Initial	Found	Calculated for 100 per cent equilibrium	Initial	Found	Calculated for 100 per cent equilibrium
Glucose monophosphate	5,060	3310	3100	12,320	10,200	9600
Glucose-1,6-diphosphate	0	3230	3100	0	10,500	9600

DISCUSSION

The activation of the phosphoglucomutase reaction by synthetic glucose-1 6-diphosphate confirms the work of Leloir and coworkers (1, 2). The apparent lack of necessity of a coenzyme in previous work in this laboratory, in particular in the work of Najjar (5) with crystalline phosphoglucomutase, was due to the presence of the coenzyme as an impurity in the glucose-1-phosphate used as substrate. Various samples of glucose-1-phosphate were found to contain from 0.03 to 0.1 per cent of glucose-1,6-diphosphate, amounts sufficient to give maximal activation of the enzyme but certainly too low to be detected by ordinary chemical analysis. The mechanism of the formation of glucose-1,6-diphosphate during the enzymatic preparation of glucose-1-phosphate is not clearly understood at the present time.

There have been several attempts to elucidate the mechanism of action of phosphoglucomutase. Meyerhof *et al.* (10) investigated the occurrence of an exchange between the glucose monophosphates and radioactive inorganic phosphate and found no exchange. Schlamowitz and Greenberg (11) investigated the possibility of an exchange of glucose (C^{14}) with glucose monophosphates in this reaction and again found no evidence of exchange. These authors postulated a mechanism which involved the intermediate formation of a compound with the phosphate attached simultaneously to

C-1 and C-6 of glucose. Recently Jagannathan and Luck (12) have reported that they observed an exchange between glucose-1-phosphate (P⁴²) and a labile phosphate group in the enzyme. However, the crystalline phosphoglucomutase used in the present study contained no measurable phosphate.

The mechanism suggested by Leloir *et al.* (2), based on glucose-1,6-diphosphate as the coenzyme of the reaction, envisages the enzyme catalyzing the transfer of the phosphate from position 1 of the coenzyme to position 6 of glucose-1-phosphate. In this way, the diphosphate becomes glucose-6-phosphate and glucose-1,6-diphosphate is regenerated from glucose-1-phosphate. In the reverse reaction, the phosphate from position 6 of the diphosphate would be transferred to position 1 of glucose-6-phosphate, yielding glucose-1-phosphate and again regenerating glucose-1,6-diphosphate. This reaction may be represented in the following way.

```
C-6-phosphate      C-1-phosphate      C-6-phosphate      C-1-phosphate
     |                   |                  |                  |
     C                   C                  C                  C
     |                   |                  |                  |
     C          +        C         ⇌        C          +       C
     |                   |                  |                  |
     C                   C                  C                  C
     |                   |                  |                  |
     C                   C                  C                  C
     |                   |                  |                  |
C-1-phosphate   →        C                  C  ←——————————  C-6-phosphate
```
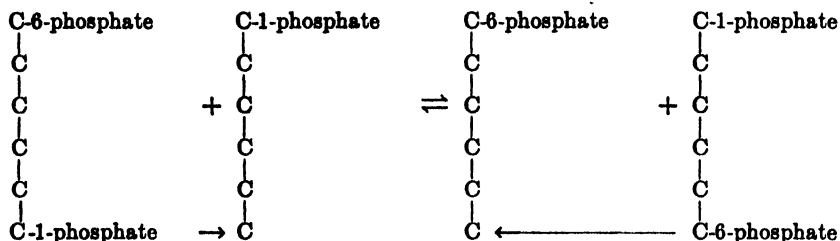
The data presented in this paper on the exchange between the phosphate group of glucose monophosphates with the phosphate groups of glucose-1,6-diphosphate, and the exchange of the glucose moiety as well, are consistent with such a mechanism. The exchange of the phosphate alone could be explained by other mechanisms but the exchange involving the carbon clearly demonstrates that the monophosphate is transformed to the diphosphate and the diphosphate, in turn, is transformed to the monophosphate.

It should be pointed out that this mode of participation of a coenzyme in a reaction, namely its conversion to substrate and its simultaneous regeneration from substrate, is unique among enzyme mechanisms studied heretofore. How generally such a mechanism occurs remains a question for further investigation. It has already been shown for the phosphoglyceric acid mutase reaction (13) that the coenzyme 2,3-diphosphoglyceric acid plays an analogous rôle to glucose-1,6-diphosphate in the phosphoglucomutase reaction.

SUMMARY

1. Certain preparations of glucose-1-phosphate, both natural and synthetic, were found to be inactive, while others showed varying rates of

conversion to glucose-6-phosphate in the reaction catalyzed by phosphoglucomutase. Inactive preparations of glucose-1-phosphate could be made fully active by the addition of catalytic amounts of synthetic α-glucose-1,6-diphosphate, 5×10^{-7} mole per liter being sufficient for half maximal rate. On this basis, samples of glucose-1-phosphate which were maximally active without the addition of glucose-1,6-diphosphate contained from 0.03 to 0.1 per cent of this cofactor; others which were not fully active contained less.

2. The mechanism of the reaction was investigated with glucose-1-phosphate labeled with C^{14} and P^{32} as substrate, crystalline phosphoglucomutase from muscle as enzyme, and non-labeled glucose-1,6-diphosphate (in this case added in large amount) as coenzyme. Complete equilibration of the phosphate and glucose moieties of the monophosphate esters and diphosphate ester occurred. This finding is consistent with a mechanism proposed by Leloir and coworkers for this reversible reaction, whereby the glucose-1,6-diphosphate transfers a phosphate group to the glucose-1-phosphate or glucose-6-phosphate. The diphosphate ester is thereby transformed to the product of the reaction and simultaneously the diphosphate is regenerated from the monophosphate. The net effect is a continuous interconversion of substrate and coenzyme, which explains the exchange.

BIBLIOGRAPHY

1. Leloir, L. F., Trucco, R. E., Cardini, C. E., Paladini, A., and Caputto, R., *Arch. Biochem.*, **19**, 339 (1948).
2. Cardini, C. E., Paladini, A. C., Caputto, R., Leloir, L. F., and Trucco, R. E., *Arch. Biochem.*, **22**, 87 (1949).
3. Posternak, T., *J. Biol. Chem.*, **180**, 1269 (1949).
4. Sutherland, E. W., *J. Biol. Chem.*, **180**, 1279 (1949).
5. Najjar, V. A., *J. Biol. Chem.*, **175**, 281 (1948).
6. Sutherland, E., Posternak, T. Z., Cori, C. F., *Federation Proc.*, **8**, 258 (1949).
7. Cori, C. F., Colowick, S. P., and Cori, G. T., *J. Biol. Chem.*, **121**, 465 (1937).
8. Doudoroff, M., Barker, H. A., and Hassid, W. Z., *J. Biol. Chem.*, **168**, 725 (1947).
9. Doudoroff, M., *J. Biol. Chem.*, **151**, 351 (1943).
10. Meyerhof, O., Ohlmeyer, P., Gentner, W., and Maier-Leibnitz, H., *Biochem. Z.*, **298**, 396 (1938).
11. Schlamowitz, M., and Greenberg, D. M., *J. Biol. Chem.*, **171**, 293 (1947).
12. Jagannathan, V., and Luck, J. M., *Federation Proc.*, **8**, 209 (1949).
13. Sutherland, E. W., Posternak, T. Z., and Cori, C. F., *J. Biol. Chem.*, **179**, 501 (1949).

A SPECIFIC COLOR REACTION OF GLYCOLIC ALDEHYDE

By ZACHARIAS DISCHE AND ELLEN BORENFREUND

(*From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University, New York*)

Glycolic aldehyde, which may play a rôle in intermediary metabolism (1, 2), has not as yet been isolated from physiological materials in the form of a well defined derivative. Its detection in animal tissues requires very sensitive specific tests. Such a test is described in this report.

EXPERIMENTAL

Simple Qualitative Test

Procedure—To 1 cc. of the solution to be tested 2 cc. of the reagent are added in test-tubes of uniform size. The reagent consists of 800 mg. of diphenylamine (commercial preparation recrystallized from 70 per cent alcohol) in 80 cc. of glacial acetic acid (Baker's Special for Shellac Analysis)[1] and 0.55 cc. of sulfuric acid (c.p., N- and As-free). The mixture is shaken vigorously, and then the tubes are immersed in boiling water for 30 minutes. Solutions containing glycolic aldehyde in concentrations of as little as 1 mg. per cent show a grass-green color.

Specificity of Reaction—Trioses show a characteristic brown color. Acetaldehyde and formaldehyde show a greenish yellow and in concentrations of above 0.005 per cent a turbidity. Fructose above 0.01 per cent produces a greenish blue color, while aldohexoses, pentoses, and methylpentoses do not show any appreciable color up to concentrations of 0.05 per cent. The green color seems to be specific for glycolic aldehyde.

Spectrophotometric Test for Glycolic Aldehyde

When glycolic aldehyde is present in mixtures which contain a great excess of other sugars or aldehydes, the test described above is not feasible. In such a case, however, it is possible to detect glycolic aldehyde spectrophotometrically.

Procedure—To 1 cc. of the unknown to be tested 0.2 cc. of a solution containing 100 gm. of trichloroacetic acid in 100 cc. of the solution and 2.4 cc. of a freshly prepared 1 per cent solution of recrystallized diphenylamine in very pure glacial acetic acid (Baker's Special for Shellac Analysis)[1]

---

[1] Unidentified impurities in other brands of glacial acetic acid give rise to colored products in the blank and influence the absorption curve of trioses, fructose, and aldehydes. It becomes more difficult under these circumstances to detect glycolic aldehyde in the presence of a large excess of these substances.

are added. The test-tubes are vigorously shaken and immersed in boiling water for 30 minutes. Solutions of glycolic aldehyde show a grass-green color, while trioses, fructose, and formaldehyde produce a blue color, which on cooling changes into bluish green. Other sugars in solutions below 0.05 per cent do not show any appreciable color.

Absorption Spectrum—As can be seen in Fig. 1, the various aldehydes and sugars differ considerably in their absorption spectra. The maximum for glycolic aldehyde is at 660 mμ, that for fructose is at 620 mμ, dihydroxyacetone at 600 mμ, glyceraldehyde at 515 mμ, and formaldehyde at
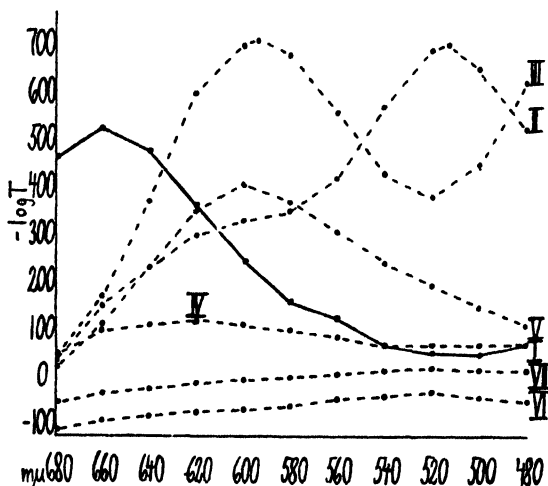
FIG. 1. Absorption spectra of various substances in the diphenylamine reaction. Curve I, glycolic aldehyde 2.5 mg. per cent; Curve II, glyceraldehyde 25 mg. per cent; Curve III, formaldehyde 2.5 mg. per cent; Curve IV, fructose 100 mg. per cent; Curve V, dihydroxyacetone 25 mg. per cent; Curve VI, ribose 100 mg. per cent; Curve VII, glucuronic acid 100 mg. per cent.

595 mμ, whereas acetaldehyde, aldopentoses, aldohexoses, and hexuronic acids show almost horizontal absorption curves between 680 and 440 mμ.

Detection of Glycolic Aldehyde in Presence of Other Aldehydic Compounds— The characteristic differences of the absorption curves enable one to detect glycolic aldehyde in the presence of a 50-fold excess of trioses and fructose or other aldehydes and sugars. To this end we determine the density of the solution at two wave-lengths, 660 and 580 mμ. As can be seen from Table I, $D_{660} - D_{580}$ is strongly positive for glycolic aldehyde but negative for all the other compounds investigated. A solution containing 1 mg. of glycolic aldehyde in 100 cc. of H_2O gives a $D_{660} - D_{580}$ of 0.150. Therefore 2 γ of glycolic aldehyde can be detected by this means. If in the unknown solution dihydroxyacetone phosphate is the only sub-

stance which interferes with the detection of glycolic aldehyde, it is more convenient to use $D_{660} - D_{500}$, which gives a slightly negative value for dihydroxyacetone phosphate and a highly positive one for glycolic aldehyde.

TABLE I

Densities at 660, 580, and 500 mμ and Their Differences for Various Substances

Substance	Concentration	D_{660} × 1000	D_{580} × 1000	D_{500} × 1000	$D_{660} - D_{580}$ × 1000	$D_{660} - D_{500}$ × 1000
	mg. per cent					
Glycolic aldehyde	2.5	+555	+180	+75	+375	+480
Formaldehyde	2.5	+185	+700	+465	−515	−280
Acetaldehyde	5.0	+8	+24	−5	−16	+13
Ribose	100.0	−72	−40	−20	−32	−52
Glyceraldehyde	25.0	+172	+370	+670	−198	−498
Xylose	100.0	−62	−30	−18	−32	−44
Fructose	100.0	+108	+120	+89	−12	+19
Glucose	1000.0	−55	−54	−20	−1	−35
Dihydroxyacetone	25.0	+130	+390	+162	−260	−32
Adenosinephosphoric acid	50.0	−23	−12	−1	−11	−22
Galacturonic acid	50.0	+34	+31	+52	+3	−18
Glucuronic acid	100.0	−19	+19	+38	−38	−57
Rhamnose	100.0	−33	−30	−18	−3	−15
Thymonucleic acid	25.0	+50	+200	+145	−150	−95
Mixture						
Fructose	200.0 ⎫					
Dihydroxyacetone	100.0 ⎬ +210	+167		+43		
Ribose	100.0 ⎭					
Glycolic aldehyde	4.0					

DISCUSSION

$D_{660} - D_{580}$ of the diphenylamine reaction of glycolic aldehyde is proportional to the concentration of this compound, and therefore under certain circumstances it should be possible to determine glycolic aldehyde quantitatively. The formation of the green compound by glycolic aldehyde depends upon the concentration of the acid in the reaction mixture. With increasing concentration of acid, beyond that used in the test, the color becomes less specific, and finally the characteristic green color is no longer produced. Therefore when glycolic aldehyde has to be determined in a solution which contains trichloroacetic acid, it is necessary to determine its amount by titration and to add correspondingly less trichloroacetic acid for the reaction itself, in order that the final concentration of this acid in the sample may be 16.7 per cent.

It is remarkable that the specificity of the diphenylamine reaction of desoxyribose nucleic acid depends upon approximately the same limits of concentration of acid as does that of glycolic aldehyde (3).

SUMMARY

1. A new characteristic color reaction for glycolic aldehyde is described.

2. A spectrophotometric method for the detection of glycolic aldehyde in the presence of an excess of other sugars and aldehydes is elaborated.

BIBLIOGRAPHY

1. Dische, Z., *Naturwissenschaften*, **26**, 252 (1938).
2. Loughran, G. A., Soodak, M., and Nord, F. F., *Arch. Biochem.*, **6**, 163 (1945).
3. Dische, Z., *Mikrochemie*, **7**, 33 (1929).

INHIBITION OF ACONITASE BY *trans*-ACONITATE[*]

By MURRAY SAFFRAN and J. LEAL PRADO[†]

(From the Departments of Biochemistry and Psychiatry, McGill University and the Allan Memorial Institute of Psychiatry, Montreal, Canada)

Bernheim (2), studying the properties of the "citric dehydrogenase" system, noted that aconitic acid (presumably the trans isomer) inhibited the reduction of methylene blue by citric acid. More recently, Morrison and Still (3), in a short report, claimed that *trans*-aconitic acid inhibited competitively the reaction *cis*-aconitate ⇌ citrate, catalyzed by the aconitase of rhubarb leaf.

The present studies show that *trans*-aconitate is a competitive inhibitor of aconitase of animal tissues and that *trans*-aconitate inhibits the respiration of tissue slices and causes the accumulation of citrate by surviving kidney cortex and liver slices.

Methods

Aconitase—The aconitase used in these studies was a water extract of pigeon breast muscle (4) prepared by homogenizing the muscle with water in a Waring blendor or in a glass homogenizer and centrifuging the homogenate at 3000 R.P.M. for 10 to 15 minutes. The cloudy supernatant, which contained most of the activity, was used. The activity of the enzyme preparation was approximately directly proportional to the mass of pigeon muscle extracted (Fig. 1). In all experiments the amount of enzyme is expressed in terms of the original weight of muscle.

The breast muscle was stored in the freezing compartment of the refrigerator. The frozen tissue retained aconitase activity for many months (4). The extracts of muscle also retained aconitase activity if kept frozen, but lost activity slowly over a period of weeks, approximately half of the activity remaining after 21 days (Fig. 2).

The extracts of frozen muscle exhibited no respiratory activity.

Estimation of Citrate—The method of Natelson et al. (5), by which citric acid is estimated but not *cis*- or *trans*-aconitic or isocitric acids, was modified for use with the standard cuvettes of the Evelyn or Coleman universal photometers.

Manometric Methods—The studies on respiration were carried out with the usual Warburg technique in phosphate-buffered media.

Chemical Preparations—*trans*-Aconitic acid was prepared from citric acid (6). *cis*-Aconitic anhydride, which yields the acid when dissolved in water, was made from *trans*-aconitic acid (7). Other substances were purified commercial preparations. The acids were neutralized with equivalent amounts of sodium bicarbonate before use.
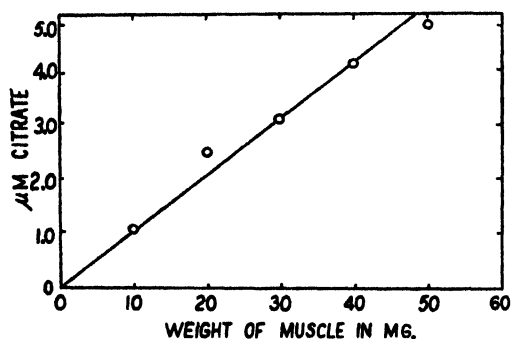
FIG. 1   FIG. 2

FIG. 1. Aconitase activity of an aqueous extract of frozen pigeon breast muscle. 10 μM of *cis*-aconitate in 1 ml. of 0.1 M phosphate buffer (pH 7.4) were incubated in open tubes with 1 ml. of an aqueous extract of 10 to 50 mg. of frozen tissue. The reaction was stopped at 30 minutes by the addition of 2 ml. of 25 per cent trichloroacetic acid and the contents of the tubes were analyzed for citrate. Temperature, 38°.
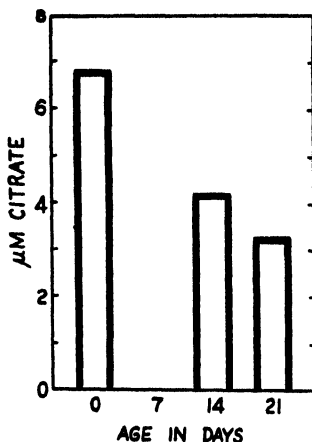
FIG. 2. Stability of frozen aconitase extracts. Conditions the same as for Fig. 1. The extract in each determination represented 67 mg. of fresh muscle.

Results

Inhibition of Aconitase—When *cis*-aconitate is incubated with aconitase, about 85 to 90 per cent is converted to citrate at equilibrium; citrate, incubated with aconitase, disappears to the extent of about 10 to 15 per cent (8). The addition of 0.06 M *trans*-aconitate to either of these systems diminishes the rate of interconversion. It also causes the reaction to stop before equilibrium is attained (Fig. 3). This may be the result of inactivation of the enzyme under the conditions of the experiment.

The extent of the inhibition depends upon the concentration of *trans*-aconitate; increasing the concentration of *trans*-aconitate results in the decreased formation of citrate from *cis*-aconitate (Fig. 4). For a large portion of the curve the inhibition is a linear function of the common logarithm of the concentration of inhibitor.

Nature of Inhibition—Prolonged exposure of aconitase to high concentrations of *trans*-aconitate (0.06 M) at 38° apparently results in inactivation of the enzyme, since the usual equilibrium is not attained (Fig. 3). The nature of the inhibition with lower concentrations of inhibitor was also investigated.
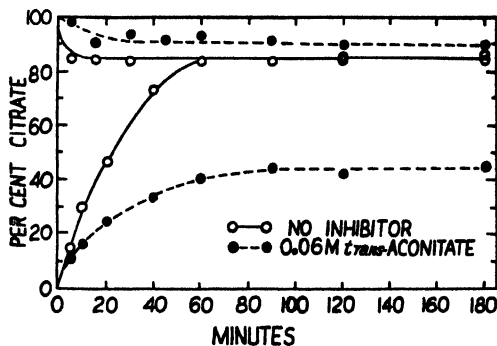
FIG. 3
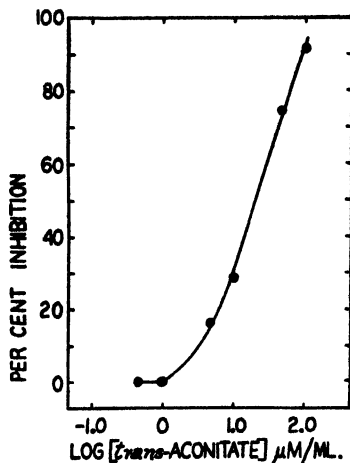
FIG. 4

FIG. 3. Inhibition of aconitase by *trans*-aconitate. Incubation carried out in open tubes containing 1 ml. of substrate (10 μM of citrate or *cis*-aconitate) in 0.1 M phosphate buffer (pH 7.4), either 1 ml. of buffer or 1 ml. of *trans*-aconitate (180 μM) in buffer, and 1 ml. of an aqueous extract of 30 mg. of frozen muscle. Temperature, 38°. Reaction stopped by the addition of 2 ml. of a 25 per cent solution of trichloroacetic acid. 1 ml. aliquots of the supernatant were analyzed for citrate.

FIG. 4. Inhibition of aconitase by increasing concentrations of *trans*-aconitate. Incubation carried out in Warburg flasks containing 0.1 ml. of *cis*-aconitate (10 μM), 2.0 ml. of *trans*-aconitate (5 to 1000 μM), both in 0.1 M phosphate buffer (pH 7.4), and 0.5 ml. of aconitase extract (4 mg. of pigeon muscle) in buffer. The reaction was stopped after 60 minutes by the addition of 2.5 ml. of a 20 per cent solution of trichloroacetic acid and the contents of the flasks were analyzed for citrate. Gas, N_2; temperature, 37°.

Lineweaver and Burk (9) have developed graphic criteria for competitive inhibition: plotting the reciprocal of the initial concentration of substrate ($1/s_0$) against the reciprocal of the velocity of the reaction ($1/v$), in the presence of increasing amounts of a competitive inhibitor, produces a family of straight lines of increasing slope, but with a common intercept on the ordinate.

Concentrations of *cis*-aconitate of 0.0001 to 0.002 M were incubated for 10 minutes with aconitase and 0, 0.005, and 0.010 M *trans*-aconitate. Plotting $1/s_0$ against $1/v$ (Fig. 5) results in three straight lines of increasing slope, but with slightly different intercepts on the ordinate. The dif-

ference between the intercepts may be explained in part by experimental error, but it may be caused by a slight amount of the inactivation of the enzyme that was seen in Fig. 3, when higher concentrations of *trans*-
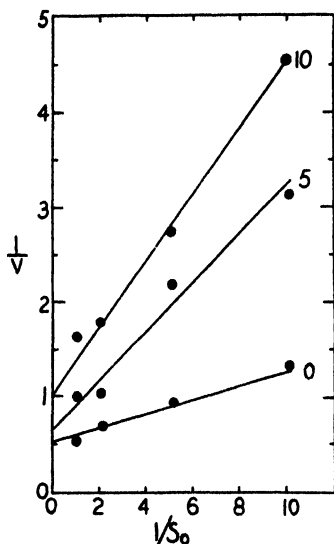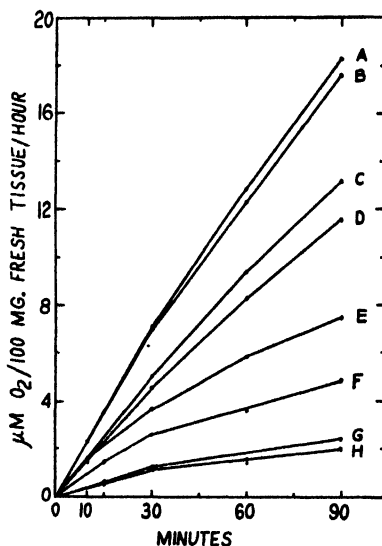
FIG. 5   FIG. 6

FIG. 5. Competitive inhibition of aconitase by *trans*-aconitate. The numerals on the curves indicate the concentration of *trans*-aconitate in micromoles per ml. v = micromoles of citrate formed in 10 minutes; s_0 = initial concentration of *cis*-aconitate in micromoles per ml. The experiment was performed in open tubes containing 0.1 to 2.0 ml. of *cis*-aconitate (1 to 20 μM) in 0.1 M phosphate buffer (pH 7.4), 1 or 2 ml. of *trans*-aconitate (50 or 100 μM) in buffer, buffer to 7.0 ml., and 3.0 ml. of an aqueous extract of frozen pigeon muscle, representing 20 mg. of tissue. Temperature, 38°. The reaction was stopped after 10 minutes by the addition of 1 ml. of a 100 per cent (weight by volume) solution of trichloroacetic acid and the contents of the tubes were analyzed for citrate.

FIG. 6. Inhibition of the respiration of rat kidney cortex slices by *trans*-aconitate and malonate. Curves A and B, normal respiration; Curve C, 0.002 M *trans*-aconitate; Curve D, 0.002 M malonate; Curve E, 0.002 M *trans*-aconitate together with 0.002 M malonate; Curve F, 0.02 M *trans*-aconitate; Curve G, 0.02 M malonate; Curve H, 0.02 M *trans*-aconitate together with 0.02 M malonate. Medium, 2 ml. of calcium-free Krebs-Ringer-phosphate solution. Gas, O_2; temperature, 37°.

aconitate were used. With lower concentrations of inhibitor the inhibition appears to be predominantly competitive.

Inhibition of Respiration—The addition of *trans*-aconitate to slices of kidney cortex or liver, respiring in calcium-free Ringer-phosphate medium, caused a definite decrease in the rate of oxygen consumption. The in-
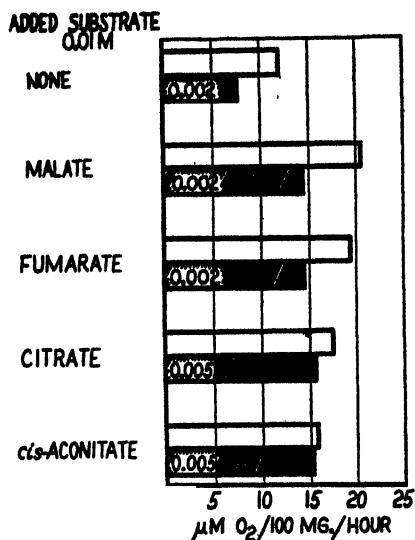
FIG. 7. Effect of the addition of compounds of the Krebs cycle on the inhibition of the respiration of slices of rat kidney cortex by *trans*-aconitate. Unshaded bars, no inhibitor added; shaded bars, respiration in the presence of the indicated concentration of *trans*-aconitate. Conditions the same as in Fig. 6.

TABLE I

Effect of Increasing Concentrations of trans-Aconitate on Respiration of Tissue Slices in Calcium-Free Ringer-Phosphate Solution

Tissue	*trans*-Aconitate added	Oxygen uptake	Inhibition
	$M \times 10^{-4}$	μM *per* 100 *mg. per hr.*	*per cent*
Rabbit kidney cortex	0	6.4	
	2	5.4	16
	20	4.6	28
	200	4.1	36
Rat kidney cortex	0	12.0	
	2	12.0	0
	20	10.2	15
	200	6.4	47
" liver	0	5.0	
	2	4.4	12
	20	3.9	22
	200	2.6	88

hibition of respiration of rat kidney cortex slices by 0.002 and 0.02 M *trans*-aconitate is compared with the inhibition by the same concentrations of malonate in Fig. 6. The inhibition of respiration was also seen

in the presence of added malate or fumarate, but was largely overcome by the addition of the tricarboxylic acids, citric and *cis*-aconitic (Fig. 7). The inhibition of respiration of slices of various tissues is increased by increasing concentrations of *trans*-aconitate (Table I).

Effect on Accumulation of Citrate—The accumulation of citrate by slices of kidney cortex and liver was greatly increased in the presence of 0.02 M *trans*-aconitate. This effect of *trans*-aconitate was enhanced by the addition of 0.01 M pyruvate or malate, but not by the addition of acetate. The results obtained with rabbit kidney cortex slices are illustrated in Fig. 8.
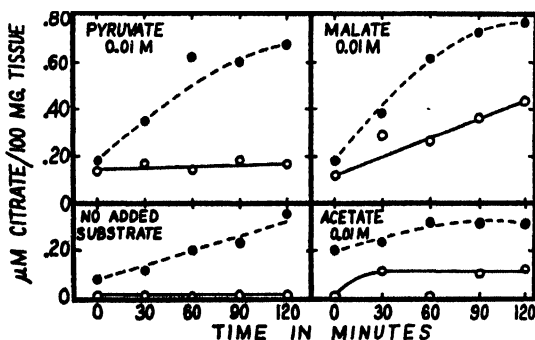
FIG. 8. Effect of *trans*-aconitate on the accumulation of citrate by slices of rabbit kidney cortex. O, no inhibitor added; ●, 0.02 M *trans*-aconitate added. Medium, 2 ml. of calcium-free Krebs-Ringer-bicarbonate solution, pH 7.4, with an atmosphere of 5 per cent CO_2 in O_2. Temperature, 37°. Tissue killed by the addition of 0.5 ml. of a 100 per cent (weight by volume) solution of trichloroacetic acid and the contents of the flasks analyzed for citrate.

DISCUSSION

Research in intermediary metabolism has elucidated many of the reactions in the Krebs or tricarboxylic acid cycle, but several gaps remain. One of the unknown regions is that involving the tricarboxylic acids, *citric*, *cis-aconitic*, and *isocitric*. Evidence from isotope studies (10–12) seemed to eliminate symmetrical compounds, like citrate, from the direct path of the Krebs cycle, and consequently citrate was placed in a side reaction. However, recent theoretical considerations by Ogston (13) have pointed out that the formation of only one optical isomer of isocitrate from citrate indicates that citrate is attached to aconitase in one way only, in effect endowing the molecule with asymmetrical properties. This argument has been strengthened by experimental evidence. Stern and Ochoa (14) reported recently that citrate was formed from oxalacetate and acetate under conditions in which aconitase could not be demonstrated.

Another experimental approach to this problem is made possible by using *trans*-aconitate as an inhibitor of aconitase. If it is assumed that one of the three tricarboxylic acids, citric, *cis*-aconitic, or isocitric, is the product of the condensation of oxalacetate and a 2-carbon compound, three schemes can be set up.

If the additional assumption is made that *trans*-aconitate specifically inhibits aconitase (indicated by the dashed lines in the schemes), the effect of the addition of *trans*-aconitate on the accumulation of citrate would depend upon the order in which the tricarboxylic acids are formed. Scheme

(A)
$$C_2 \searrow \quad C_4 \nearrow \rightarrow \text{Citrate} \rightleftharpoons \text{cis-Aconitate} \rightleftharpoons \text{Isocitrate} \downarrow \text{Krebs cycle}$$

(B)
$$\text{Citrate}$$
$$C_2 \searrow \quad C_4 \nearrow \rightarrow \text{cis-Aconitate} \rightleftharpoons \text{Isocitrate} \downarrow \text{Krebs cycle}$$

(C)
$$\text{Citrate}$$
$$\text{cis-Aconitate}$$
$$C_2 \searrow \quad C_4 \nearrow \rightarrow \text{Isocitrate} \downarrow \text{Krebs cycle}$$
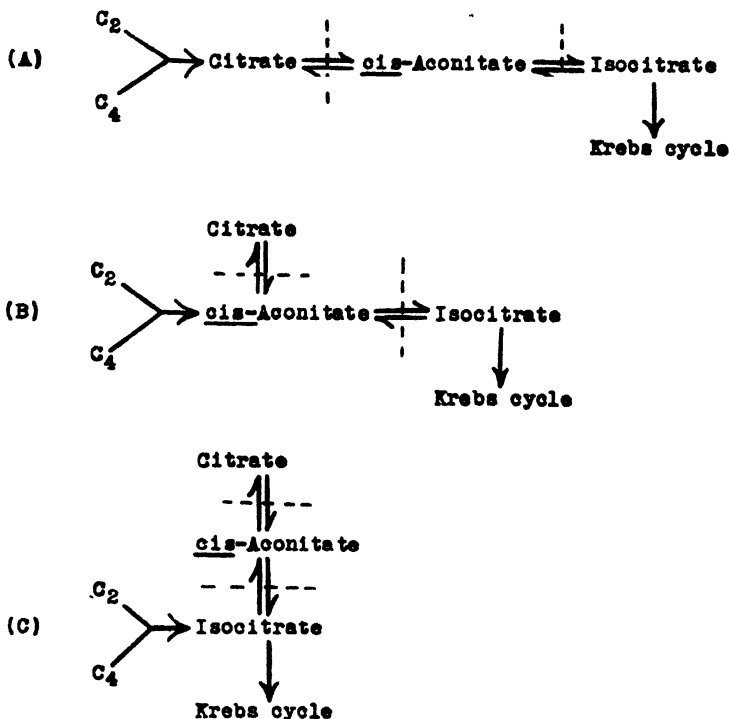
A would result in the accumulation of more citrate in the presence than in the absence of the inhibitor, because the breakdown of citrate would be inhibited. Scheme B would result in the accumulation of less citrate because its formation would be inhibited. Scheme C would drastically reduce the amount of citrate formed and would not explain the inhibition of respiration by *trans*-aconitate.

In this investigation citrate accumulated in larger amounts when *trans*-aconitate was added to tissue slices, suggesting that Scheme A is probably involved. However, similar results might be expected from Scheme B if

trans-aconitate inhibits the reaction *cis*-aconitate $\rightleftharpoons$ isocitrate more strongly than the reaction *cis*-aconitate $\rightleftharpoons$ citrate, implying the existence of the two aconitases of Jacobson *et al.* (15). Alternatively, these results could be explained by all three schemes if the further breakdown of isocitrate were inhibited more strongly by *trans*-aconitate than the reactions of the aconitase system (Scheme D).

Experiments to test the effect of *trans*-aconitate on the reaction *cis*-aconitate $\rightleftharpoons$ isocitrate and on the breakdown of isocitrate are now in progress.

We are indebted to Dr. K. A. C. Elliott and Dr. O. F. Denstedt for their interest and encouragement in this investigation.

(D)

SUMMARY

1. The inhibition of aconitase by *trans*-aconitate is primarily competitive. Prolonged exposure to high concentrations of inhibitor apparently results in inactivation of the enzyme.

2. The respiration of kidney cortex and liver slices is inhibited by *trans*-aconitate. This inhibition is largely overcome by the addition of citrate or *cis*-aconitate.

3. The accumulation of citrate by tissue slices is markedly increased by *trans*-aconitate, suggesting that citrate is formed by the condensation of oxalacetate and a 2-carbon compound.

4. The accumulation of citrate from acetate by slices of rabbit kidney cortex is much less than from pyruvate or malate.

BIBLIOGRAPHY

1. Saffran, M., and Prado, J. L., *Federation Proc.*, **7**, 182 (1948).
2. Bernheim, F., *Biochem. J.*, **22**, 1178 (1928).
3. Morrison, J. F., and Still, J. L., *Australian J. Sc.*, **9**, 150 (1947).
4. Krebs, H. A., and Eggleston, L. V., *Biochem. J.*, **38**, 426 (1944).
5. Natelson, S., Lugovoy, J. K., and Pincus, J. B., *J. Biol. Chem.*, **170**, 597 (1947).
6. *Org. Syntheses*, coll. **2**, 12 (1943).

7. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 185 (1945).

8. Martius, C., and Leonhardt, H., *Z. physiol. Chem.*, **278**, 208 (1943).

9. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, **56**, 658 (1934).

10. Evans, E. A., Jr., and Slotin, L., *J. Biol. Chem.*, **141**, 439 (1941).

11. Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 31 (1942).

12. Weinhouse, S., and Millington, R. H., *J. Am. Chem. Soc.*, **69**, 3089 (1947).

13. Ogston, A. G., *Nature*, **162**, 963 (1948).

14. Stern, J. R., and Ochoa, S., *J. Biol. Chem.*, **179**, 491 (1949).

15. Jacobson, K. P., Soares, M., and Tapadinhas, J., *Bull. Soc. chim. biol.*, **22**, 48 (1940).

INDEX TO AUTHORS

L

M

●

INDEX TO SUBJECTS